Recent Advances in Phytochemistry 44

Reinhard Jetter *Editor*

Phytochemicals – Biosynthesis, Function and Application

Volume 44

Springer

Phytochemicals—Biosynthesis, Function and Application

Recent Advances in Phytochemistry

Editorial Board

For further volumes:
http://www.springer.com/series/6474

Reinhard Jetter
Editor

Phytochemicals—Biosynthesis, Function and Application

Volume 44

Springer

Editor
Reinhard Jetter
Departments of Botany and Chemistry
The University of British Columbia
Vancouver
British Columbia
Canada

ISBN 978-3-319-04044-8 ISBN 978-3-319-04045-5 (eBook)
DOI 10.1007/978-3-319-04045-5
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014931750

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Preface to the 44th Volume of the Recent Advances in Phytochemistry Series

Welcome to the fourth volume since the reintroduction of the *Recent Advances in Phytochemistry* (*RAP*) series, an annual series championed by the Phytochemical Society of North America. *RAP* is dedicated to publishing both review and primary research articles for a broad audience of biologists, chemists, biochemists, pharmacologists, clinicians and nutrition experts, especially those interested in the biosynthesis, structure, function and/or bioactivity of plant natural products. Recurring themes include the evolution and ecology of specialized metabolites, the genetic and enzymatic mechanisms for plant products formation and metabolism, the systems biology study of cells, tissues, and organs, the engineering of plant natural products, as well as various aspects of their application for human health. Also, new developments in the techniques used to study plant natural products are presented and discussed, for example, those for structure elucidation and quantification, for "omic" (genomic/proteomic/transcriptomic/metabolomics) profiling or for microscopic localization. In short, this series combines chapters from experts that will explain and discuss selected topics, to highlight the most exciting new research in phytochemistry.

Two main types of articles are printed in *RAP*: Perspectives and Communications. The *RAP* Perspectives aim to give a general introduction to a field and an overview of the pertinent literature as a backdrop for new results from the primary literature. These articles may be similar to review articles, but often present important ideas and hypotheses, including proposals for interesting directions in the field. It is the hope of the Editorial Board that these articles will be of great value to a large audience. The *RAP* Communications focus more on primary data that showcases particular new advances in a specialized field that will be of interest to a large audience. Articles of both types are typically solicited from prominent members of the Phytochemical Society of North America and based on the content of the annual meeting talks. However, the Editorial Board also invites additional Perspectives and/or Communications from selected authors beyond the society's meeting to give a rounded picture of all "Recent Advances in Phytochemistry".

All submissions to *RAP* go through a rigorous, external peer review process, overseen by the Editorial Board. Since the reintroduction of the series, *RAP* is indexed together with all journals published by Springer. All *RAP* papers are available not only in the published volume form, but also electronically through Springer's

online literature services. This marks a significant change from older volumes of *RAP*, and it is the hope of the Editorial Board that this will lead to broader dissemination and greater interest in *RAP*.

This 44th volume of *RAP* includes a total of eight articles mostly based on talks presented at the 51st annual meeting of the Phytochemical Society of North America. As was seen in *RAP* volumes 41–43, these Perspectives give a very good picture of the breadth of plant (bio)chemistry research in North America, which is also indicative of the state of the field worldwide. Each of these articles describes the integration of several different approaches to ask and then answer questions regarding the function of interesting plant metabolites, either in the plant itself or in interactions with the environment (natural setting or human health application).

Two Perspectives focus on the biosynthesis of natural products: Dastmalchi and Dhaubhadel summarize new findings on the formation and regulation of isoflavonoids, using soybean seeds as a model system. In contrast, Cook et al. give an overview of natural product formation by microorganisms associated with plants, with particular emphasis on alkaloids from the locoweeds.

The next two chapters give updates on the bioactivity of selected plant natural products. Timoshenko et al. highlight progress on toxic lectins from various plant species such as mistletoes and elderberries. Guerrero-Analco et al., on the other hand, summarize work on plant natural products with activity against type 2 diabetes, taking an ethno-botanical approach combining Aboriginal knowledge with modern phytochemistry.

Two further Perspectives feature biotechnological approaches, both starting with genomic and biochemical results and exploring their applications in metabolic engineering. This modern branch of Phytochemistry is explored by Zerbe and Bohlmann in their chapter on terpenoid products from conifers and also by Lisko et al. in their review of vitamin C formation in various plant species.

Finally, two more Perspectives further highlight analytical aspects of plant natural products. Berhow et al. review the composition of camelina oil, a very promising commodity containing a wide variety of plant lipids, while Glover and Murch focus on the qualitative and quantitative analysis of a potentially toxic amino acid found in tropical food chains.

Overall, we are excited to present this broad set of review papers on various aspects of modern phytochemistry. We hope you will find these Perspectives to be interesting, informative and timely. It is our goal that *RAP* will act not only as the voice of the Phytochemical Society of North America, but that it will serve as an authoritative, up-to-date resource that helps to set the gold standard for thought and research in plant natural products. Enjoy the read!

The RAP Editorial Board

Vancouver, BC — Reinhard Jetter
Ottawa, ON — John T. Arnason
Pullman, WA — David R. Gang
Corvallis, OR — J. Fred Stevens
Blacksburg, VA — Dorothea Tholl

Contents

Contributors

Siddique I. Aboobucker Arkansas Biosciences Institute, Arkansas State University, Arkansas, USA

John T. Arnason Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

Deniz Belenli Faculty of Veterinary Medicine, Department of Biochemistry, University of Uludag, Bursa, Turkey

Mark A. Berhow United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Functional Foods Research Unit, Peoria, Illinois, USA

Paleah Black Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Jörg Bohlmann Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

Daniel Cook Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, UT, United States

Alain Currier Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Institut de recherche en biologie végétale, Jardin botanique de Montréal, Université de Montréal, Montréal, QC, Canada

Mehran Dastmalchi Department of Biology, The University of Western Ontario, London, ON, Canada

Sangeeta Dhaubhadel Department of Biology, The University of Western Ontario, London, ON, Canada

Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, ON, Canada

Hoda M. Eid Department of Pharmacology, Université de Montréal, Montréal, QC, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

Hans-Joachim Gabius Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany

Dale R. Gardner Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, UT, United States

W. Broc Glover Department of Chemistry, University of British Columbia, Kelowna, BC, Canada

Irina V. Gorudko Department of Biophysics, Belarusian State University, Minsk, Belarus

Daniel Grum Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, UT, United States

José A. Guerrero-Analco Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Pierre S. Haddad Department of Pharmacology, Université de Montréal, Montréal, QC, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

Katherine A. Lisko Arkansas Biosciences Institute, Arkansas State University, Arkansas, USA

Argelia Lorence Department of Chemistry and Physics, Arkansas State University, Arkansas, USA

Arkansas Biosciences Institute, Arkansas State University, Arkansas, USA

Louis C. Martineau Department of Pharmacology, Université de Montréal, Montréal, QC, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

Bryan R. Moser United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Bio-Oil Research Unit, Peoria, Illinois, USA

Asim Muhammad Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Susan J. Murch Department of Chemistry, University of British Columbia, Kelowna, BC, Canada

Lina Musallam Department of Pharmacology, Université de Montréal, Montréal, QC, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

James A. Pfister Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, UT, United States

Umit Polat Faculty of Veterinary Medicine, Department of Biochemistry, University of Uludag, Bursa, Turkey

Ammar Saleem Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Nan Shang Department of Pharmacology, Université de Montréal, Montréal, QC, Canada

Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines, Montréal, QC, Canada

Montreal Diabetes Research Centre, Université de Montréal, Montréal, QC, Canada

Alexander V. Timoshenko Department of Biology, Western University, London, Ontario, Canada

Raquel Torres Arkansas Biosciences Institute, Arkansas State University, Arkansas, USA

Steven F. Vaughn United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Functional Foods Research Unit, Peoria, Illinois, USA

Philipp Zerbe Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

Chapter 1
Soybean Seed Isoflavonoids: Biosynthesis and Regulation

Mehran Dastmalchi and Sangeeta Dhaubhadel

Abstract Isoflavonoids are plant natural products, almost exclusive to legumes, synthesized by the phenylpropanoid pathway. They are actors in symbiosis with nitrogen-fixing bacteria and involved in plant pathogen and stress response. Isoflavonoids are noted for their wide range of human health benefits. Isoflavonoids, as phytoestrogens, can bind to estrogen receptors and modulate their activity in animals including humans. Soybean seeds contain three isoflavone aglycones that are glycosylated and/or malonylated and stored in vacuoles. The biosynthetic pathway starts with the recruitment of phenylalanine and enters its first committed branch step with the conversion of flavanone to isoflavone. Soybean seeds accumulate large amounts of isoflavonoids as a result of *de novo* synthesis and transport. The isoflavonoid content and composition in the seed are complex polygenic traits that are highly variable. Environmental factors, including drought, light conditions, fertilization, temperature and CO_2 levels, and genetic factors, such as specific pathway gene members, transcription factors coordinating expression and conjugating enzymes, have all been shown to have an effect on isoflavonoid content. Understanding the genetic and molecular basis for isoflavonoid biosynthesis and its regulatory mechanisms will allow manipulation of content in soybean seeds and metabolic engineering of isoflavonoids in nonleguminous plants.

Keywords Isoflavonoids · Soybean · Transcription factors · Seed · Natural product · Gene regulation

S. Dhaubhadel (✉) · M. Dastmalchi
Department of Biology, The University of Western Ontario,
London, ON N6A 5B7, Canada

S. Dhaubhadel
Southern Crop Protection and Food Research Centre,
Agriculture and Agri-Food Canada, 1391 Sandford Street,
London, ON N5V 4T3, Canada
e-mail: sangeeta.dhaubhadel@agr.gc.ca

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application*,
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_1

1.1 Introduction

Isoflavonoids are plant natural products synthesized by the phenylpropanoid pathway. They accumulate almost exclusively in plant species belonging to the Fabaceae family. Isoflavonoids have recently been reported at relatively lower levels in some nonlegume plants [1, 2]. Isoflavonoids are integral to plant interactions with their environment, as precursors to phytoalexins, which function against a variety of pests and plant pathogens, and as signaling molecules in symbiosis with nitrogen-fixing bacteria [3–5]. Soybean (*Glycine max* [L.] Merr.) seeds contain large amounts of isoflavonoids and are the primary source of these metabolites in human food and animal feed. There is considerable epidemiologic and clinical evidence highlighting the positive health effects associated with isoflavonoid consumption by animals including humans, through various forms of intake, such as soy foods and nutritional supplements [6–8].

In this chapter, we begin by discussing the role of isoflavonoids in human health (Sect. 1.2.1) and their role in interactions between plants and the environment (Sect. 1.2.2); we will then expand on the chemical structure and diversity of isoflavonoids (Sect. 1.3.1), their biosynthesis (Sect. 1.3.2), and accumulation (Sect. 1.3.3), followed by a discussion on the regulation of isoflavonoid biosynthesis in soybean seeds reviewing the genetic (Sect. 1.4.1) and environmental factors (Sect. 1.4.2); finally we draw conclusions on current findings and outline future directions for studying isoflavonoid biosynthesis and regulation (Sect. 1.5).

1.2 Biological Roles

1.2.1 Roles in Human Health and Nutrition

Soybean has been a staple food of Asian diets for centuries that, beyond representing an essential source of dietary protein, has been linked with the disparity of health and longevity that exists between Asian and Western populations [9–12]. In particular, the significantly lower rates of hormone-dependent cancers in Asian countries such as Japan and China [13] have been seen as a corollary of increased soy intake. Scientists have found that isoflavonoids are the key biologically active compounds in soybean seeds associated with positive health benefits in animals including humans. These unique bioactive components in soy foods contribute to human health and specifically reduce the risk of hormone-dependent cancers [7, 12].

In the past 40 years, clinical and epidemiological studies have revealed that isoflavonoids can function in humans as phytoestrogens [7, 14]. The physiological and chemopreventive benefits of these plant natural products stem from the structural similarity of the isoflavone aglycone, genistein, to 17β-estradiol, the major endogenous form of estrogenic hormone in both females and males [8, 14] (Fig. 1.1). Genistein elicits weak estrogenic effects by competition with 17β-estradiol for

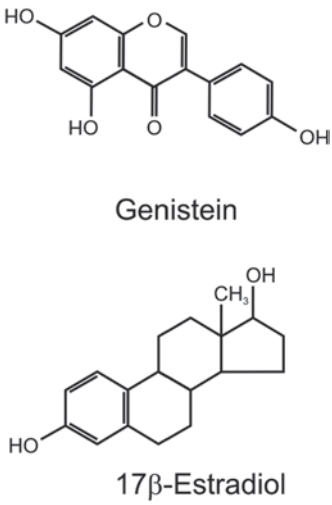


Fig. 1.1 Chemical structures of the isoflavone genistein and 17β-estradiol

binding with intranuclear estrogen receptor (ER) α, β, and sex hormone-binding globulins; preferentially binding to ERβ (reviewed in [15]). Through this form of competition, isoflavones can render a modulating activity by exerting estrogenic agonist or antagonistic effects, in the presence of low and high endogenous estrogen concentrations, respectively. The estrogenic activity of isoflavones can vary depending on the tissue and the type of ER in the cell [16].

There is a wealth of data that purports the usage of soy foods and isoflavonoid supplements in reducing the risk of breast cancer in women and prostate cancer in men, based on *in vitro* studies, animal models, and clinical trials [9, 12, 17]. Epidemiological evidence suggests that soy consumption during early childhood or adolescence yields 28–60 % reduction in the risk of developing breast cancer [18–20]. Dietary genistein blocks activation of the human prostate cancer cell marker, p38 mitogen-activated protein kinase, inhibiting the downstream metastasis and cell invasion cascade [21]. Yet another possible function for genistein in humans is as a tyrosine kinase inhibitor that interferes with HIV-mediated actin dynamics and thereby inhibits HIV infection [22]. Isoflavonoids are also underlined as the key components in climacteric medicine. Intake of isoflavonoids has been suggested to ease such post-menopausal ailments in women as osteoporosis [23] and hot flashes [24]. Furthermore, dietary intake of isoflavonoids has been linked with reduction in the risk of cardiovascular disease and high blood cholesterol levels [25, 26].

There is some controversy regarding the strength of the link drawn between dietary consumption of isoflavonoids and human health benefits. Studies have been criticized for a high degree of heterogeneity among trials, arbitrarily determined soy intake levels and variation in phytoestrogen dosage. This has led some observers to believe that evidence for a causal link between isoflavonoid intake and its benefits are inconsistent or misleading [12]. These concerns must be addressed with an established standard of phytoestrogen dosages between studies and longer clinical

trials. Additionally, dietary intake of isoflavonoids for infants and other hormonally susceptible groups has raised some concerns. In particular, the usage of soy-based infant formula has been problematic despite several decades of continuous use in North America [15]. Questions are mainly centered on the possible effects of isoflavonoid accumulation and increase in estrogenic activity. The intake of soy formula in infants, considering their body mass, produces doses of isoflavonoids that are much higher than in adults eating soy foods; however, no adverse developmental or health effects have been reported [27].

Concisely, isoflavonoids are plant natural products that have a wide range of human health benefits. Owing to their stereochemical capability to bind to estrogen receptors and modulate estrogenic activity, they are thought to reduce the risk of hormone-dependent cancers. The controversy attached to this very estrogenic activity creates a challenge to the soy-based food industries to adapt for different dietary requirements. Therefore, from a health and nutritional prospective, seed isoflavonoid content requires much more research to understand its biosynthesis and regulation, in anticipation of developing soybeans with altered isoflavonoid levels that fulfil consumer requirements.

1.2.2 Roles in Interaction Between Plants and Environment

Isoflavonoids are of dual importance in plant interactions with their environment: as phytoalexins in basal and innate responses to pathogens and stress, and as signals in symbiosis with nitrogen-fixing bacteria.

Phytoalexins are low-molecular weight, host-induced antimicrobial substances that are released in response to pathogen attack or stress. Phytoalexins constitute a broad-spectrum plant defense against both prokaryotic and eukaryotic microorganisms [28]. Soybean phytoalexins include both the simple isoflavones and their more complex derivatives. Several studies have reported the rapid increase in isoflavonoid levels upon pathogen attack in soybean[29–31]. The synthesis of isoflavonoids, particularly the pterocarpan glyceollins or active aglycones such as daidzein, formononetin, and genistein (reviewed in [32]), during defense response is controlled both by the host [33] and the pathogen [34]. Synthesis and release of phytoalexins can limit pathogen colonization, induce toxicity, and increase plant resistance. The synthesis and function of glyceollin have been extensively studied. This pterocarpan phytoalexin inhibits the growth of a wide range of soybean pathogens such as *Phytophthora sojae, Sclerotinia sclerotiorum,* and *Macrophomina phaseolina* [35]. This has been supported experimentally by silencing isoflavone synthase (IFS) and chalcone synthase (CHS) expression, which reduced the levels of isoflavone daidzein, a glyceollin precursor, and decreased levels of glyceollin, leading to increased susceptibility to pathogens including the root rot oomycete *P. sojae* [36, 37] and the fungal pathogen *Fusarium virguliforme* [35].

The rapid accumulation of glyceollin and other phytoalexins is imperative to limiting colonization by pathogens. The speed of synthesis is important, as pathogens might be capable of degrading phytoalexins, avoiding or limiting the efficacy

of these plant defense compounds. Higher ability to degrade or modify phytoalexins, such as glyceollin, into innocuous compounds in *Diaprothe phaseolorum* var. *meridionales* and *Rhizoctonia solani,* results in faster growth [35]. The challenge for soybean breeding would be to firstly, enhance the production of glyceollin in response to pathogens, and secondly, to prevent degradation of phytoalexins by pathogens. This would improve soybean resistance to a broad range of plant pathogen infections.

Another form of plant-environment interaction mediated by isoflavonoids is the symbiosis between legumes and nitrogen-fixing bacteria. Atmospheric nitrogen represents a vast inaccessible resource for plants. Legumes have developed symbioses with nitrogen-fixing rhizobia to circumvent this problem [38]. Isoflavonoids are key factors in this interaction as signaling molecules that induce rhizobial nodulation genes, leading to bacterial attachment to root hair cells of the legume plant [39]. The cascade of events that follows includes, invagination of the root hair cell by a bacterial infection thread and a concurrent development of the root nodule, a specialized organ that allows for an exchange of fixed nitrogen in the form of ammonia from the rhizobia [40]. Subramanian et al. [5] provided the first genetic evidence that endogenous isoflavonoids are essential for soybean root nodule formation. Treatment of soybean roots with *Bradyrhizobium japonicum* induced the expression of the key isoflavonoid biosynthetic enzyme IFS, in roots. Evidence for the involvement of isoflavonoids in root nodulation came from RNA interference (RNAi) silencing of IFS genes in soybean hairy root composite plants, which led to severely reduced nodulation [5].

The legume-rhizobial symbiotic relationship mediated by isoflavonoids is of great importance for its ability to acquire nitrogen indirectly from the atmosphere, and to reduce the economic and environmental burden imposed by nitrogenous fertilizers.

1.3 Isoflavonoid Synthesis and Accumulation

1.3.1 Structure and Diversity

The isoflavone aglycones are the most bioactive forms of isoflavonoids with phytoestrogenic capability in animals including humans [14]. Soybean seeds contain nine different isoflavonoids (Fig. 1.2): the three core isoflavone aglycones daidzein, genistein, and glycitein, their corresponding 7-O-β-D glycosides (daidzin, genistin, and glycitin), and 6″-O-malonyl-7-O-β-D glycosides (malonyldaidzin, malonylgenistin, and malonylglycitin) [41]. The malonyl glycosides are thermally unstable and, as a result of processing, are converted into their corresponding acetylglycosides (6″-O-acetyldaidzin, 6″-O-acetylgenistin, and 6″-O-acetylglycitin) commonly found in soy food products, such as tofu and soy sauce [42]. Glycosylation and malonylation of soybean isoflavones allows their compartmentalization into vacuoles for storage owing to their stability and solubility. The amount of each isoflavone varies considerably in soybean seeds, but in the majority of cases they

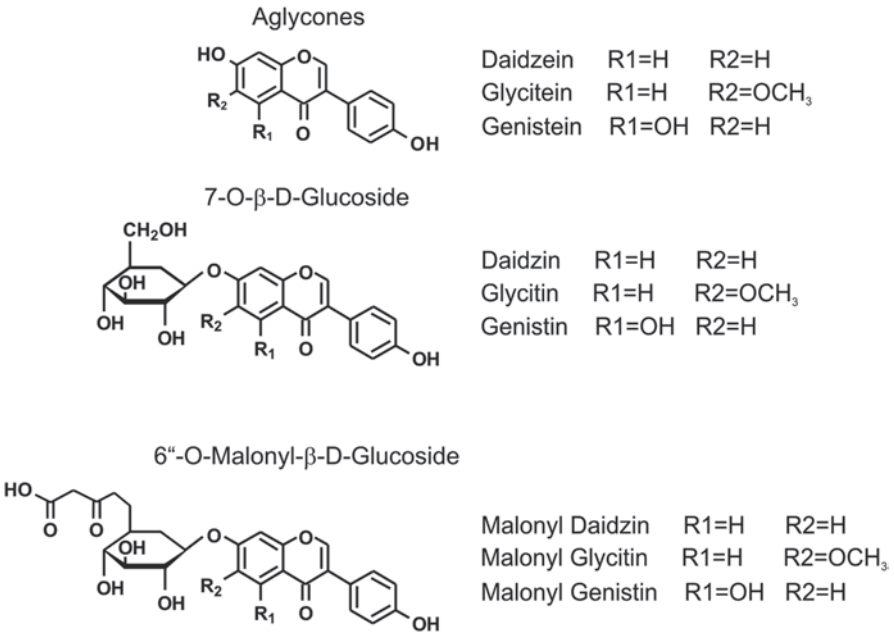


Fig. 1.2 Structure of nine major isoflavonoids synthesized in soybean seeds

are found in the ratio of 4:5:1 (daidzein: genistein: glycitein) [43]. Despite of their highly unstable nature, the majority of soybean seed isoflavonoids accumulate in the form of their malonyl derivatives (Fig. 1.3). It appears that the plant vacuoles contain an unknown mechanism(s) that confers stability to the malonyl isoflavone conjugates *in planta*.

1.3.2 Biosynthesis

Isoflavonoids are synthesized through a legume-specific branch of the phenylpropanoid pathway (Fig. 1.4). Phenylpropanoids are found throughout the plant kingdom and include a plethora of secondary metabolites, among which are flavonoids, condensed tannins, monolignols, and stilbenoids. Isoflavonoid biosynthesis branches off with the conversion of flavanones to isoflavones by the activity of IFS. There is evidence for the protein–protein interaction between several of the key phenylpropanoid pathway enzymes with the membrane-bound P450s (reviewed in [44–46]). It has been hypothesized that the isoflavonoid biosynthetic enzymes may form a dynamic complex, the isoflavonoid metabolon, giving rise to a higher degree of complexity for their manipulation in plants.

The general phenylpropanoid pathway begins with the recruitment and subsequent deamination of phenylalanine into cinnamate by the enzyme phenylalanine ammonia-lyase (PAL), producing the general phenylpropanoid skeleton. Catalytic activity of

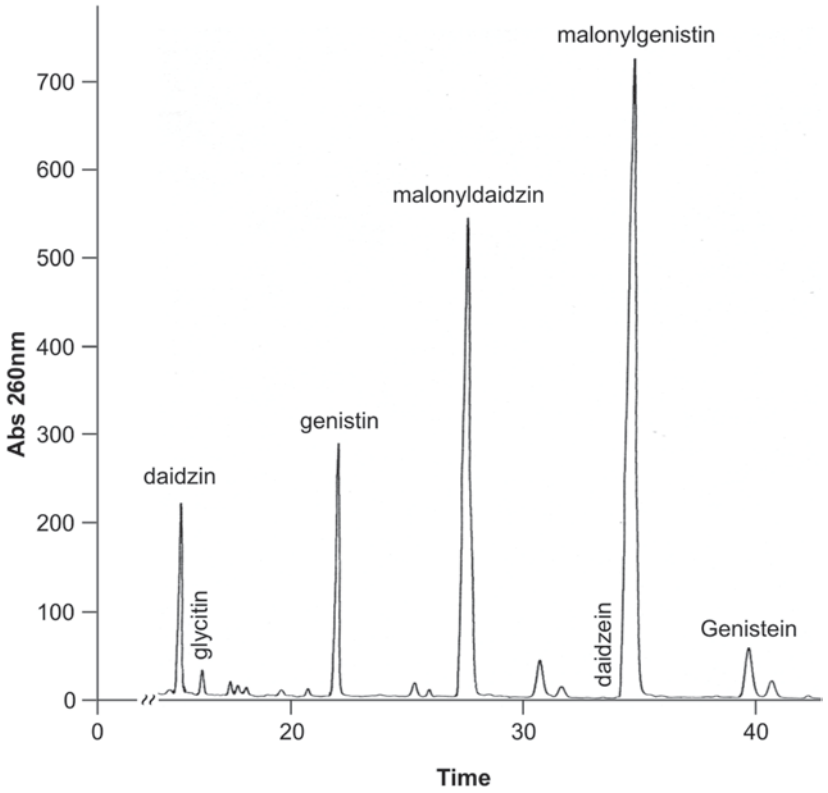


Fig. 1.3 HPLC analysis of isoflavonoids extracted from mature soybean seeds

PAL is directly related to the efficiency of the entire pathway and the downstream biosynthesis of secondary metabolites, as it commits the flux of primary metabolites into a plethora of secondary metabolites [47]. Plant PAL structures possess a conserved fold with a central core formed by near-parallel α-helices [48], which resemble histidine ammonia-lyase (HAL) [49]. It is postulated that PAL and the phenylpropanoid pathway evolved from the histidine degradation pathway and HAL, with the advent of plant annexation of land and the divergence of fungi and plants from other kingdoms. The reaction product of PAL, cinnamate, is converted into *p*-coumarate by cinnamate 4-hydroxylase (C4H). C4H is a cytochrome P450 monooxygenase and is membrane-bound. The last step of the general phenylpropanoid pathway is the conversion of *p*-coumarate to *p*-coumaroyl-CoA by 4-coumarate-CoA-ligase.

As shown in Fig. 1.4, the first committed step in the flavonoid and isoflavonoid pathways is the formation of a chalcone, by CHS, via the condensation of three malonyl-CoA molecules and a *p*-coumaroyl-CoA. All flavonoids and isoflavonoids are built from a chalcone scaffold, which is biosynthesized by the pivotal, plant-specific polyketide synthase CHS. It directs the intramolecular cyclization of the coumaroyl moiety and three acetates to form a polyketide. CHS usually functions as a homodimer, with each monomer containing two structural domains, an upper

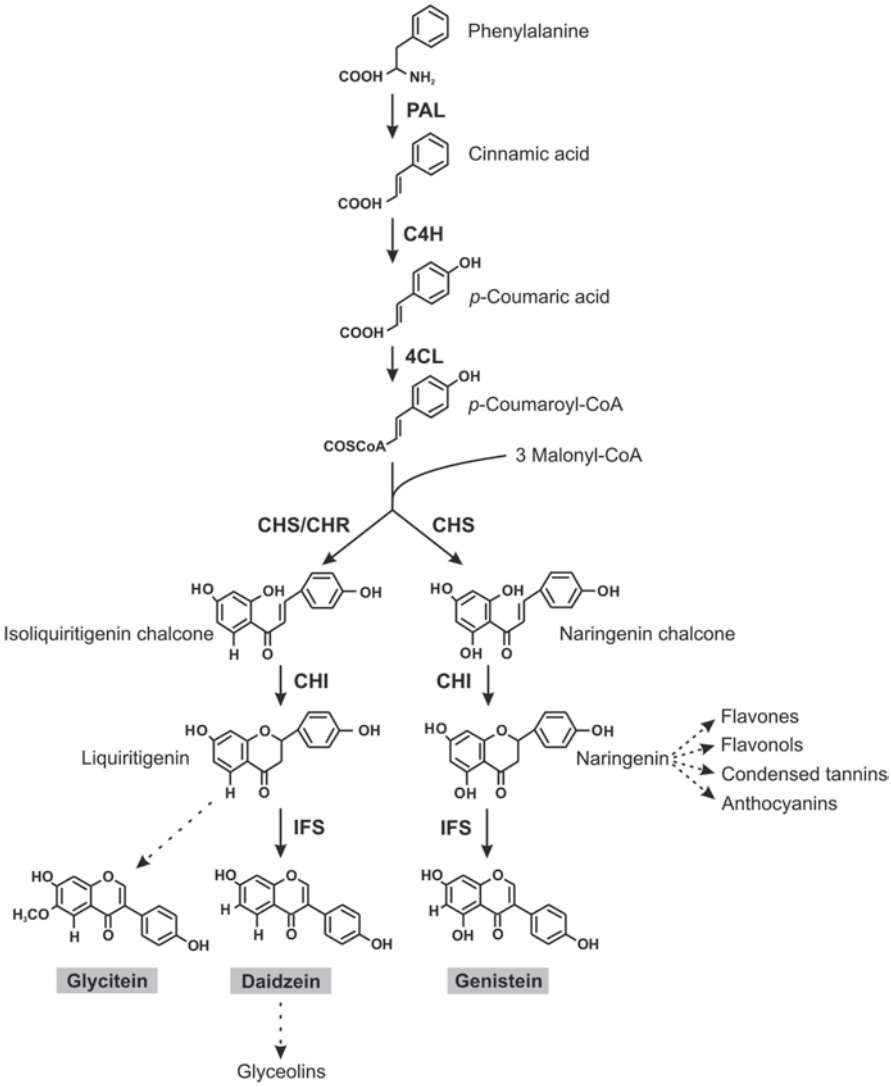


Fig. 1.4 Schematic diagram of the phenylpropanoid pathway leading to the production of isoflavonoids in soybean. *Dotted arrows* indicate multiple steps. PAL phenylalanine ammonia lyase, 4CL 4-coumarate-CoA-ligase, C4H cinnamate-4-hydroxylase, CHI chalcone isomerase, CHR chalcone reductase, CHS chalcone synthase, IFS 2-hydroxyisoflavanone synthase

and lower domain, forming a cleft where the active site is situated [50]. The soybean genome contains nine CHS genes (CHS1–CHS9), including a duplicated copy of CHS1. The soybean CHS gene family shares a high degree of sequence similarity, ranging from 80 to 99% nucleotide sequence identity in the coding region. The members of the CHS gene family are expressed differentially during plant development and/or in response to environmental cues. Among the CHS genes, CHS7

and CHS8 are critical for isoflavonoid biosynthesis and accumulation in soybean [51, 52]. The end product of a CHS-catalyzed reaction is a bicyclic tetrahydroxy chalcone (naringenin chalcone). Leguminous plants produce an additional trihydroxy chalcone (isoliquiritigenin chalcone) through the coupled activity of CHS and the legume-specific chalcone reductase (CHR). CHR regiospecifically reduces coumaroyl-trione, a polyketide intermediate of the CHS reaction to an alcohol. The product of the CHS/CHR coaction, isoliquiritigenin, allows legumes to produce novel-derived compounds, such as daidzein, medicarpin, and glyceollin [53, 54].

The bicyclic chalcone scaffolds, naringenin chalcone and isoliquiritigenin chalcone, are the substrates for the second catalytic step in the isoflavonoid pathway catalyzed by chalcone isomerase (CHI), producing the tricyclic flavanones naringenin and liquiritigenin, respectively [53]. The dichotomy of the flavonoid and isoflavonoid pathways is thought to be established by the presence of multiple CHIs in plants with varying catalytic abilities. The CHI family has been categorized into two types based on their catalytic abilities: type I CHIs, common to all higher plants, convert naringenin chalcone to naringenin, and type II CHIs, almost exclusive to legumes, have the additional capacity to catalyze the conversion of isoliquiritigenin to liquiritigenin [55].

The versatility of legume type II CHIs and their direct role in isoflavonoid production have led to speculation over their necessity for achieving significant isoflavonoid yields during metabolic engineering efforts in plants [56, 57]. The soybean CHI gene family has eight members, categorized into four subfamilies: CHI1, CHI2, CHI3 and CHI4. There are four amino acid residues (Thr48, Tyr106, Asn113, and Thr190) in type II CHIs that are critical for the legume-specific catalysis of isoliquiritigenin to liquiritigenin [58]. A comparison of the deduced amino acid sequences of the soybean CHI1 and CHI2 subfamilies and type II CHIs in other legumes shows that the critical residues lining the active sites are almost entirely conserved, except for the substitution of Thr190 in CHI2, which reduces its catalytic efficiency by six fold [58]. The two subfamilies CHI3 and CHI4 do not have any conservation of the critical active site residues, which explains their inability to convert any of the chalcone derivatives used in yeast expression assays [55].

CHI enzymes were previously thought to exist exclusively in the plant kingdom [59]; however, recent studies have found structural homologs containing the CHI-unique open-faced β-sandwich fold in bryophytes, fungi, and bacteria [60]. The catalytic residues and hence their catalytic abilities can vary largely, and many species containing a biologically active CHI lack homologs of CHS, and vice versa [60]. Therefore, it seems that the evolution of CHI and the phenylpropanoid pathway appeared gradually in land plants. Further research will be required to understand the function of CHI in fungal and bacterial species, and to characterize soybean CHI subfamilies lacking the catalytic ability to produce flavanones. Enzymes such as CHI, carrying a wide variation and long evolutionary timeline, can help elucidate the origin and evolution of flavonoid and isoflavonoid biosynthesis.

As shown in Fig. 1.4, flavanones (naringenin and liquiritigenin) are the precursors that lead to the production of a range of plant secondary metabolites including anthocyanins, condensed tannins, flavonoids, and phlobaphenes. In legumes, the

branch point leading to isoflavonoid production is the conversion of flavanones into isoflavones by IFS, also known as 2-hydroxyisoflavanone synthase (2-HIS) [61–63]. IFS is a legume-specific, membrane-bound cytochrome P450 monooxygenase that catalyzes a C-2 to C-3 aryl-ring migration and hydroxylation in position C-2. The reaction consumes reduced nicotinamide adenine dinucleotide phosphate and an oxygen molecule, producing the unstable intermediate 2-hydroxyisoflavanone, which undergoes dehydration with the help of 2-hydroxyisoflavanone dehydratase to form the double bond between C2-C3, and produces the isoflavone aglycones: daidzein, genistein, and glycitein, in soybean. Two IFS genes, IFS1 and IFS2, have been found in soybean, and both have the catalytic ability to mediate the production of isoflavones [62, 63]. IFS1 and IFS2 differ by 14 amino acid residues and show differential tissue-specific expression in soybean [64, 65]. IFS has been proven necessary and sufficient for introducing isoflavonoid production in nonlegumes, albeit only at low levels [47, 56, 57, 66, 67].

Soybean isoflavone aglycones can undergo conjugation, resulting in their corresponding glycoside and malonyl-glycoside derivatives. Conjugation with a glucose or malonyl-glucose molecule confers the aglycones with enhanced water solubility and reduced chemical reactivity, thereby altering the physiological activity and allowing their proper storage in cell vacuoles [68]. Addition of the glycosyl and malonyl moieties to the isoflavone aglycones is mediated by isoflavonoid-specific uridine diphosphate glycosyltransferase (UGT) and malonyltransferase (MT), respectively. Glycosylation involves the transfer of a nucleotide diphosphate-activated sugar donor molecule onto an aglycone acceptor molecule [68]. UGTs are multifunctional enzymes, with a wide range of possible acceptor molecules *in vivo*, including flavonols, isoflavones, and chalcones [69]. Glycosylation is the common final step in the biosynthesis of most flavonoids, reducing cytotoxicity of high levels of volatile, hydrophobic flavonols. Further conjugation by malonylation adds a malonyl group from malonyl-CoA to the glycosyl moiety of isoflavone glycosides, thereby protecting them from glycosidases [70]. In soybean, isoflavonoid-specific UGT73F2 and GmMT7/GmIF7MaT are involved in glycosylation and malonylation of the core isoflavone aglycones, respectively [71, 72]. The overall function of the conjugations appears to be the enhancement of compartmentalization to the central vacuole of the cell and subsequent transport out of the cell (Fig. 1.5). Conjugation is therefore imperative for the role of isoflavonoids and their derivative compounds in plant interaction with the environment, including induction of nodulation genes in symbiotic nitrogen-fixing bacteria and as phytoalexins.

1.3.3 Accumulation

Isoflavonoids accumulate in all organs of soybean plants, but their levels vary depending on the tissue type and developmental stage. The highest concentrations of isoflavonoids are found in mature seeds (Fig. 1.6) and leaves [64]. Soybean embryos express all the key biosynthetic genes involved in isoflavonoid biosynthesis, and excised embryos have an ability to take up exogenously supplied isoflavonoids,

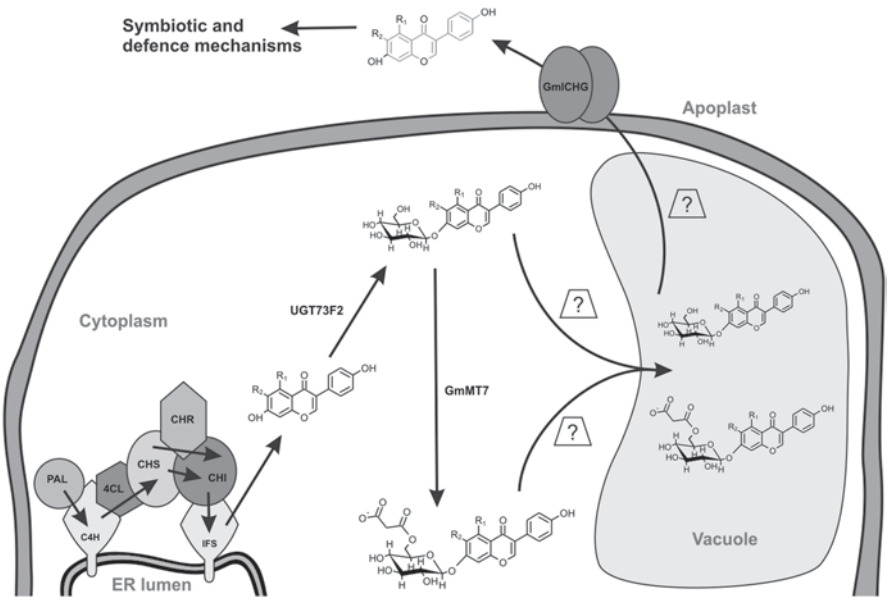


Fig. 1.5 Diagram showing phenylpropanoid pathway enzymes involved in isoflavonoid synthesis. Subcellular location of the enzymes, formation of isoflavone metabolon and proposed regulation of soybean isoflavone conjugate pool are depicted. Isoflavone conjugates are stored in the plant cell vacuole until they are required by the plant for symbiosis or defense mechanisms. When required by the plant, the stored isoflavonoids get translocated to the apoplast, followed by cleavage of the conjugate group with the help of isoflavonoid-specific β-glucosidase (GmICHG) resulting in the release of the bioactive isoflavone aglycones. The mechanism of isoflavonoid translocation in and out of the vacuole is not known and indicated by the question mark (?)

demonstrating that these metabolites are produced locally in the tissue where they accumulate [64]. However, there is strong evidence indicating that many plant natural products are transported from their site of synthesis to the site of accumulation. For example, glucosinolates in *Brassica* species are transported into seeds from their production sites [73, 74]. Similarly, nicotine is synthesized in roots and gets transported to leaves in tobacco [75]. Therefore, it was not surprising that soybean seeds accumulate the largest amount of isoflavonoids, since the developing seed serves as a sink organ for many products synthesized in other plant tissues. In case of soybean isoflavonoids, the following indirect evidence suggests the long-distance transport of isoflavonoids within the plants: (a) embryos excised from soybean seeds accumulated isoflavonoids from a synthetic medium, (b) a maternal effect on seed isoflavonoid level was observed in the reciprocal crosses between two soybean cultivars with different seed isoflavonoid levels [64, 76], and (c) [^{14}C] malonylgenistin and malonyldaidzin accumulated in mature embryos and leaves when fed through the stem [76]. However, the long-distance transport of isoflavone glucosides has not been demonstrated directly yet. Taken together, total isoflavonoid accumulation in soybean seed is a result of both *de novo* synthesis within the seed and transport from maternal tissues [64].

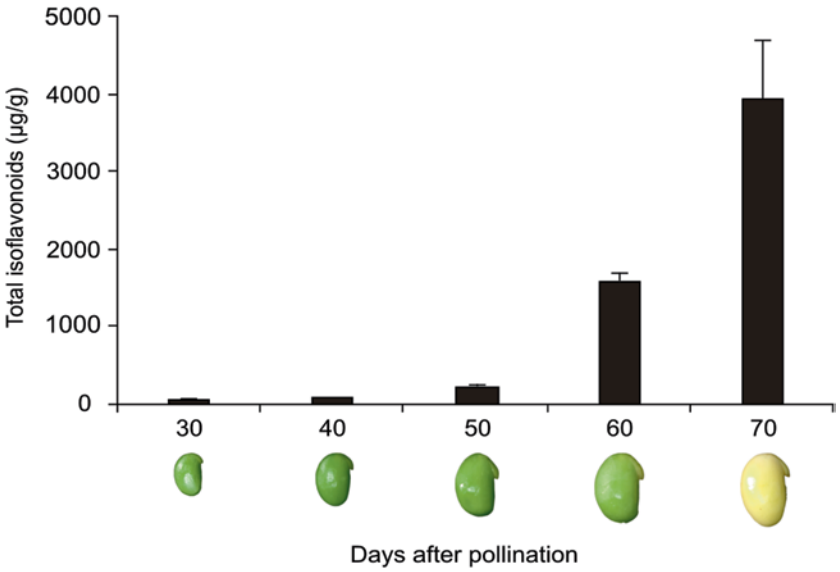


Fig. 1.6 Isoflavonoid accumulation in soybean embryos during seed development. Data shown are mean values from three independent experiments. Numbers indicate the age of embryos in days after pollination. Photographs of embryos 30–70 days after pollination are shown

1.4 Regulation of Isoflavonoid Biosynthesis in Soybean Seeds

The isoflavonoid content in soybean is a complex polygenic trait that is highly variable. Several studies have indicated that the level of isoflavonoids in soybean tissues depends both on environmental factors and the genetic makeup of the plant [77–80]. The complexity of isoflavonoid accumulation in soybean seeds has been a challenge to breeders attempting to tailor content to various consumer needs and improve overall crop quality and yield, while reducing environmental damage. The main factors that influence isoflavonoid content and their composition in soybean are discussed below.

1.4.1 Genetic Factors

Although isoflavonoids had been reported in soybean and other legumes for several decades, studies on the genetics and metabolic engineering of isoflavonoids in soybean and nonlegume plants began only after the discovery of the entry-point enzyme IFS in soybean [62, 63]. As shown in Figs. 1.4 and 1.5, isoflavonoid synthesis involves a multistep process where the pathway intermediates are channelled by several enzymes in a multienzyme complex. The synthesis of core isoflavones

requires two membrane-bound cytochrome P450 monooxygenases (C4H and IFS) that are associated with the endoplasmic reticulum [81].

There is a large degree of variation in seed isoflavonoid content among the many different soybean cultivars. Comparison of isoflavonoid accumulation in soybean seeds in different cultivars showed that high isoflavonoid cultivars consistently perform better in terms of isoflavonoid level compared to low isoflavonoid cultivars, if grown in the same environment at the same time. For example, accumulation of isoflavonoids in low isoflavonoid cultivar "Hardin" and the high isoflavonoid cultivar "Corsoy-79" were 460 and 800 μg/g of seed, respectively, when grown in Girard, Illinois. The same cultivars, Hardin and Corsoy-79, when grown in Urbana accumulated 820 and 1,550 μg isoflavonoid/g of seed, respectively [82]. To identify the genetic factor(s) that may contribute to the increase in isoflavonoid content in soybean seeds, a global gene-expression analysis was performed, and expression of isoflavonoid-related genes were compared between developing embryos isolated from two soybean cultivars, RCAT-Angora and Harovinton, that differed in the level of seed isoflavonoids [51]. The analysis included 430 genes involved either in the shikimic acid or phenylpropanoid pathways, and identified two CHS genes, CHS7 and CHS8, with significantly higher expression in RCAT-Angora (the high isoflavonoid cultivar) compared to Harovinton (the low isoflavonoid cultivar).

Of the five CHS genes (CHS1, CHS2, CHS6, CHS7, and CHS8) present on the array, only CHS7 and CHS8 showed differential expression, suggesting a critical role for these two most closely related CHS genes in isoflavonoid synthesis. RNAi silencing of CHS8 in soybean hairy roots resulted in reduced expression of both CHS7 and CHS8 genes as well as isoflavonoid level in the roots, providing direct evidence for the involvement of these two genes in isoflavonoid synthesis [52]. The CHS7 and CHS8 genes are expressed differentially in soybean tissues. A detailed analysis of CHS7 and CHS8 promoter regions for *cis*-acting factors and potential transcription factor binding sites showed structural diversity within the promoters of these two genes that may lead to their differential spatial, temporal, and stimulus-response expression. Furthermore, the results also led to the speculation that CHS7 may function in induced isoflavonoid production, whereas CHS8 is possibly involved in isoflavonoid synthesis under normal conditions [52].

The phenylpropanoid pathway is one of the most extensively studied biosynthetic pathways in plants. Studies have shown the coordinated transcriptional control of phenylpropanoid biosynthetic genes as the major mechanism dictating the level of end products (reviewed in [83]). It is well established in plants such as *Arabidopsis*, maize, and petunia that transcriptional regulation of many phenylpropanoid structural genes are regulated by the coordinated control of myeloblastosis (MYB), basic helix-loop-helix (bHLH) transcription factors, and WD40 domain proteins [84]. A chimeric transcription factor from maize, CRC, that contained R myc-type transcription factor inserted between the DNA-binding and activation domains of C1 MYB transcription factor, when expressed ectopically, was able to increase isoflavonoid content significantly in soybean seeds [46]. This led to the speculation that MYB transcription factors may regulate isoflavonoid synthesis in soybean.

A functional genomic approach in combination with gene-expression analysis in developing soybean embryos [51] identified one such transcription factor, GmMYB176, which regulates expression of CHS8 [85]. GmMYB176 is a novel R1 MYB transcription factor with SHAQKYF motif that recognizes a TAGT (T/A)(A/T) sequence in the CHS8 promoter and regulates its expression. RNAi silencing of GmMYB176 in soybean hairy roots reduced the expression of GmMYB176 and its target gene CHS8, thereby reducing the isoflavonoid level in the roots and providing direct evidence for the role of GmMYB176 in isoflavonoid synthesis [85].

GmMYB176 is the first R1 MYB transcription factor identified to have a role in plant secondary metabolism. This study also suggested the possibility of combinatorial regulation of CHS8 gene, as overexpression of GmMYB176 in hairy roots did not increase both expression of CHS8 and isoflavonoid levels [85]. Several GmMYB176 recognition motifs are present in both IFS gene promoters. RNAi silencing of GmMYB176 reduced the level of IFS expression, suggesting that GmMYB176 co-regulates multiple isoflavonoid biosynthetic genes in soybean. Overall, it has been demonstrated that the interaction between GmMYB176 and the CHS8 promoter is necessary but not sufficient for CHS8 gene activation and an increase in isoflavonoid levels [85].

The subcellular localization of GmMYB176 was investigated by making a translational fusion of GmMYB176 with yellow fluorescent protein (YFP) followed by its transient and stable expression in tobacco epidermal cells and *Arabidopsis*, respectively. In both systems, GmMYB176 localized predominantly to the nucleus, as expected given its role as a transcription factor. Despite its nuclear localization, GmMYB176 does not contain a putative nuclear localization signal, which led to the speculation that a protein–protein interaction may be involved in targeting it to the nucleus. Computational motif search analysis predicted three potential pST-binding sites within GmMYB176 to which 14-3-3 proteins may bind, thereby regulating its subcellular localization. Deletion of one of the pST-binding sites within GmMYB176 indeed affected its subcellular localization [85]. Using bimolecular fluorescent complementation and targeted yeast two-hybrid assays, it has been determined that GmMYB176 interacts with soybean 14-3-3 protein, SGF14d [86]. Plant 14-3-3s are thought to be involved in a variety of signaling processes [87–91]. They regulate activities of a variety of target proteins via protein–protein interactions, which involves binding with phosphoserine/phosphothreonine residues in the target [92, 93] or by other less studied mechanisms [94, 95]. The soybean genome contains 18 14-3-3 genes, among which 16 are transcribed [96]. 14-3-3 proteins interact with their clients in a dimer form, which allows them to act as scaffolds by bringing two different regions of the same proteins or two different proteins closer together. Interaction of GmMYB176 with 14-3-3 protein regulates its intracellular localization, thus affecting target gene expression and isoflavonoid biosynthesis [97]. The involvement of 14-3-3 protein in the regulation of the R1 MYB (GmMYB176) and plant secondary metabolism is a novel biological and biochemical phenomenon.

As discussed in Sect. 1.3.2, isoflavone aglycones are hydrophobic molecules that are toxic to the cell that produces them. Therefore, these aglycones need to be conjugated with sugar and/or malonyl-sugar groups, with the help of their corresponding

transferases, for their proper compartmentalization into plant vacuoles. Metabolic engineering of isoflavonoids in *Arabidopsis* by introducing the IFS gene was successful in producing genistein at very low levels [56]. In this case, the genistein was conjugated with endogenous sugars, glucose and rhamnose. Failure to accumulate higher levels of isoflavonoids in *Arabidopsis* could be due to the absence of isoflavonoid-specific UDP-glucuronosyltransferases (UGTs) and malonylltransferases (MTs). The legume-specific aglycones may be poor substrates for endogenous nonlegume UGTs, possibly resulting into the fast turnover of isoflavone aglycones in *Arabidopsis*. Therefore, isoflavonoid-specific UGTs and MTs might have a critical role in the accumulation of these compounds in plants.

1.4.2 Environmental Factors

Many lines of evidence strongly suggest that isoflavonoid accumulation in soybean seeds is controlled by several environmental factors [82, 98–101]. A comparison of gene expression in developing embryos of two soybean cultivars that contrasted in the level of seed isoflavonoids demonstrated that the environmental effects on gene expression were large and exceeded cultivar-specific differences [51]. The analysis included the embryo samples collected from soybean plants grown at two different locations for two consecutive years. A similar study that included a mapping population of recombinant inbred lines grown at four different environments supported the previous finding by Dhaubhadel and team [51], and suggested that seed isoflavonoid levels are determined by a complex network of several minor-effect loci interconnected by a dense epistatic map of interactions that is dependent on environmental conditions [99]. Environmental factors that influence isoflavonoid content include drought [102], light condition [103], fertilization [101, 104], temperature and CO_2 levels [100, 104–106].

1.5 Concluding Remarks and Future Directions

Developing new soybean cultivars with both high and low levels of isoflavonoids has gained considerable research interest, both for soybean seeds for human health and nutrition and for roots because of their dual function in establishing symbioses and inhibiting plant pathogens. Isoflavonoid content is a complex trait that is regulated by a dense interweaving network of genetic and environmental factors that has so far been elusive toward manipulation [99]. This complexity poses a scientific and economic challenge that can only be resolved by understanding each step of the isoflavonoid biosynthetic pathway, the factors that regulate the pathway, and the integration of environmental components in this network. Due to its agronomic importance, significant progress has been achieved in understanding the biosynthesis of isoflavonoids. Metabolic engineering approaches, either to increase the level of isoflavonoids in soybean or to introduce these compounds in nonlegumes,

have identified the bottlenecks for isoflavonoid synthesis. The major challenge has been channelling the pathway intermediates to the final product, enabled by the metabolon. Since the phenylpropanoid pathway radiates into many other pathways that produce a wide array of metabolites, it is critical to understand crosstalk between the different branches of the phenylpropanoid pathway. The results may lead us toward finding a mechanism to manipulate a specific branch of the pathway exclusively. Therefore, detailed knowledge of how the various pathways are regulated and how the metabolon is formed and used, either in flavonoid or isoflavonoid biosynthesis, is essential. Regulation of flux through the pathway may be affected by post-transcriptional regulation of enzymes or their regulators directly by phosphorylation, or through a shift in redox potential indirectly. Future studies should aim at the identification of the regulatory factors that control the expression of isoflavonoid-specific genes, the co-factors that mediate the regulation, and the mechanisms by which the regulatory factors interplay with each other to provide the overall spatiotemporal regulation of the entire isoflavonoid pathway.

References

1. Lapcík O (2007) Isoflavonoids in non-leguminous taxa: A rarity or a rule? Phytochemistry 68:2909–2916
2. Reynaud J, Guilet D, Terreux R, Lussignol M, Walchshofer N (2005) Isoflavonoids in non-leguminous families: an update. Nat Prod Rep 22:504–515
3. Dixon RA, Achnine L, Kota P, Liu CJ, Reddy MSS, Wang L (2002) The phenylpropanoid pathway and plant defence–A genomics perspective. Mol Plant Path 3:371–390
4. Phillips DA, Kapulnik Y (1995) Plant isoflavonoids, pathogens and symbionts. Trends Microbiol 3:58–64
5. Subramanian S, Stacey G, Yu O (2006) Endogenous isoflavones are essential for the establishment of symbiosis between soybean and *Bradirhizobium japonicum*. Plant J 48:261–273
6. Cederroth CR, Nef S (2009) Soy, phytoestrogens and metabolism: a review. Mol Cell Endocrinol 304:30–42
7. Dixon RA (2004) Phytooestrogen. Annu Rev Plant Biol 55:225–261
8. Dixon RA, Ferreria D (2002) Genistein. Phytochemistry 60:205–211
9. Duffy C, Perez K, Partridge A (2007) Implications of phytoestrogen intake for breast cancer. Cancer J Clin 57:260–277
10. Lamartiniere CA (2000) Protection against breast cancer with genistein: a component of soy. Am J Clin Nutr 71:1705S–1707S
11. Limer JL, Speirs V (2004) Phyto-oestrogens and breast cancer chemoprevention. Breast Cancer Res 6:119–127
12. Messina M (2010) Insights gained from 20 years of soy research. J Nutr 140:2259S–2295S
13. Pisani P, Bray F, Parkin MD (2002) Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. Intl J Cancer 97:72–81
14. Folman Y, Pope GS (1969) Effect of norethisterone acetate, dimethylstilboestrol, genistein and coumestrol on uptake of [3H] oestradiol by uterus, vagina and skeletal muscle of immature mice. J Endocrinol 44:213–218
15. Chen AM, Rogan WJ (2004) Isoflavones in soy infant formula: a review of evidence for endocrine and other activity infants. Annu Rev Nutr 24:33–54
16. Doerge DR, Sheehan DM (2002) Goitrogenic and estrogenic activity of soy isoflavones. Environ Health Perspect 110:349–353

17. Lamartiniere CA, Moore JB, Brown NM, Thompson R, Hardin MJ, Barnes S (1995) Genistein suppresses mammary cancer in rats. Carcinogenesis 16:2833–2840
18. Gutierrez-Gonzalez JJ, Wu X, Zhang J, Lee JD, Ellersieck M, Shannon JG, Yu O, Nguyen HT, Sleper DA (2009) Genetic control of soybean seed isoflavone content: importance of statistical model and epistasis in complex traits. Theor Appl Genet 119:1069–1083
19. Igarashi D, Ishida S, Fukazawa J, Takahashi Y (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. Plant Cell 13:2483–2497
20. Korde LA, Wu AH, Fears T, Nomura AM, West DW, Kolonel L, Pike MC, However R, Ziegler RG (2009) Childhood soy intake and breast cancer risk in Asian American women. Cancer Epidemiol Biomarkers Prev 18:1050–1059
21. Libault M, Joshi T, Benedito VA, Xu D, Udvardi MK, Stacey G (2009) Legume transcription factor genes; what makes legumes so special? Plant Physiol 151:991–1001
22. Guo J, Xu X, Rasheed TK, Yoder A, Yu D, Liang H, Yi F, Hawley T, Jin T, Ling B, Wu Y (2013) Genistein interferes with SDF-1- and HIV-mediated actin dynamics and inhibits HIV infection of resting CD4 T cells. Retrovirology 10:62
23. Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JW (1998) Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am J Clin Nutr 68:1375S–1379S
24. Howes LG, Howes JB, Knight DC (2006) Isoflavone therapy for menopausal flushes: a sytematic review and meta-analysis. Maturitas 55:203–211
25. Strom BL, Schinnar R, Ziegle EE, Barhard KT, Sammel MD, Macones GA, Stallings VA, Drulis JM, Nelson SE, Hanson SA (2001) Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. J Am Med Assoc 286:807–814
26. Walker HA, Dean TS, Sanders TAB, Jackson G, Ritter JM, Chowienczyk PJ (2001) The phytoestrogen genistein produces acute nitric oxide-dependent dilation of human forearm vasculature with similar potency to 17 beta-estradiol. Circulation 103:258–262
27. Mendez MA, Anthony MS, Arab L (2002) Soy-based formulae and infant growth and development: a review. J Nutr 132:2127–2130
28. Paxton JD (1981) Phytoalexins-a working redefinition. Phytopathologische Zeitschrift 101:106–109
29. Graham TL (1991) Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol 95:594–603
30. Graham TL (1995) Cellular biochemistry of phenylpropanoid responses of soybean to infection by *Phytophthora sojae*. In: Daniel M, Purkayanstha RP (eds) Handbook of phytoalexin metabolism and action. Marcel Dekker, New York, pp 85–116
31. Lozovaya VV, Lygin AV, Zernova OV, Li S, Hartman GL, Widholm JM (2004) Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. Plant Physiol Biochem 42:671–679
32. Jeandet P, Clement C, Courot E, Cordelier S (2013) Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. Intl J Mol Sci 14:14136–14170
33. Hsieh MC, Graham TL (2001) Partial purification and characterization of a soybean beta-glucosidase with high specific activity towards isoflavone conjugates. Phytochemistry 58:995–1005
34. Rivera-Vargas LI, Schmitthenner AF, Graham TL (1993) Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. Phytochemistry 32:851–857
35. Lygin AV, Hill CB, Zernova OV, Crull L, Widholm JM, Hartman GL, Lozovaya VV (2010) Response of soybean pathogens to glyceollin. Phytopathology 100:897–903
36. Graham TL, Graham MY, Subramanian S, Yu O (2007) RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in *Phytophthora sojae* infected tissues. Plant Physiol 144:728–740
37. Subramanian S, Graham MA, Yu O, Graham TL (2005) RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. Plant Physiol 137:1345–1353
38. Mulligan JT, Long SR (1985) Induction of *Rhizobium meliloti* nodC expression by plant exudate requires nodD. Proc Natl Acad Sci USA 82:6609–6613

39. Phillip DA (1992) Flavonoids: plant signals to soil microbes. In: Stafford HA, Ibrahim RK (eds) Recent advances in phytochemistry: phenolic metabolism in plants. Plenum Press, New York, pp 201–223
40. Spaink HP (2000) Root nodulation and infection factors produced by rhizobial bacteria. Annu Rev Microbiol 54:257–288
41. Kudou S, Fleury Y, Welt D, Magnolato D, Uchida T, Kitamura K (1991) Malonyl isoflavone glycosides in soybean seeds (Glycine max Merill). Agric Biol Chem 55:2227–2233
42. Griffith AP, Collison MW (2001) Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography-mass spectrometry. J Chromatogr 913:397–413
43. Wang H, Murphy PA (1994) Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 42:1674–1677
44. Jørgensen K, Rasmussen AV, Morant M, Nielsen AH, Bjarnholt N, Zagrobelny M, Bak S, Møller BL (2005) Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr Opin Plant Biol 8:280–291
45. Winkel BSJ (2004) Metabolic channeling in plants. Annu Rev. Plant Biol 55:85–107
46. Yu O, McGonigle B (2005) Metabolic engineering of isoflavone biosynthesis. Adv Agron 86:147–190
47. Liu RR, Hu YL, Li HL, Lin ZP (2007) Production of soybean isoflavone genistein in non-legume plants via genetically modified secondary metabolism pathway. Metab Eng 9:1–7
48. Ritter H, Shulz GE (2004) Structural basis for the entrance into phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase. Plant Cell 16:3426–3436
49. Shwede TF, Retey J, Shulz GE (1999) Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. BioChemistry 38:5355–5361
50. Ferrer JL, Jez JM, Bowman ME, Dixon RA, Noel JP (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Mol Biol 6:775–784
51. Dhaubhadel S, Gijzen M, Moy P, Farhangkhoee M (2007) Transcriptome analysis reveals a critical role of CHS*7* and CHS*8* genes for isoflavonoid synthesis in soybean seeds. Plant Physiol 143:326–338
52. Yi J, Derynck MR, Chen L, Dhaubhadel S (2010) Differential expression of CHS*7* and CHS*8* genes in soybean. Planta 231:741–753
53. Bednar RA, Hadcock JR (1988) Purification and characterization of chalcone isomerase from soybeans. J Biol Chem 263:9582–9588
54. Wang X (2011) Structure, function, and engineering of enzymes in isoflavonoid biosynthesis. Funct Integr Gen 11:13–22
55. Ralston L, Subramanian S, Matsuno M, Yu O (2005) Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases. Plant Physiol 137:1375–1388
56. Liu CJ, Blount JW, Steele CL, Dixon RA (2002) Bottlenecks for metabolic engineering of isoflavone glycoconjugates in Arabidopsis. Proc Natl Acad Sci USA 99:14578–14583
57. Shih C, Chen Y, Wang M, Chu IK, Lo C (2008) Accumulation of isoflavone genistin in transgenic tomato plants overexpressing a soybean isoflavone synthase gene. J Agric Food Chem 56:5655–5661
58. Jez JM, Bowman ME, Noel JP (2002) Role of hydrogen bonds in the reaction mechanism of chalcone isomerase. Biochemistry 41:5168–5176
59. Jez JM, Bowman ME, Noel JP (2000) Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. Nat Struct Mol Biol 7:786–791
60. Gensheimer M, Mushegian A (2004) Chalcone isomerase family and fold: no longer unique to plants. Protein Sci 13:540–544
61. Akashi T, Aoki T, Ayabe S-i (1999) Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiol 121:821–828

62. Jung W, Yu O, Lau SMC, O'Keefe DP, Odell J, Fader G, McGonigle B (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol 18:559–559
63. Steele CL, Gijzen M, Qutob D, Dixon RA (1999) Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. Arch Biochem Biophys 367:146–150
64. Dhaubhadel S, McGarvey BD, Williams R, Gijzen M (2003) Isoflavonoid biosynthesis and accumulation in developing soybean seeds. Plant Mol Biol 53:733–743
65. Subramanian S, Hu X, Lu GH, Odell JT, Yu O (2004) The promoters of two isoflavone synthase genes respond differentially to nodulation and defense signals in transgenic soybean roots. Plant Mol Biol 54:623–639
66. Sreevidya VS, Rao CS, Sullia SB, Ladha JK, Reddy PM (2006) Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. J Exp Botany 57:1957–1969
67. Yu O, Jung W, Shi J, Croes RA, Fader GM, McGonigle B, Odell JT (2000) Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol 124:781–794
68. Jones P, Vogt T (2001) Glycosyltransferases in secondary plant metabolism: transquilizers and stimulant controllers. Planta 213:164–174
69. Li L, Modolo LV, Escamilla-Trevino LL, Achnine L, Dixon RA, Wang X (2007) Crystal structure of *Medicago truncatula* UGT85H2-insight into structural basis of a multifunctional (iso) flavonoid glycosyltransfer-ase. J Mol Biol 370:951–963
70. Suzuki H, Nakayama T, Yonekura-Sakakibara Y, Fukui Y, Nakamura N, Yamaguchi M, Tanaka Y, Kusumi T, Nishino T (2002) cDNA cloning, heterologous expression, and functional characterization of malonyl-coenzyme A: anthocyanidin 3-O-glucoside-6-O-malonyltransferase from dahlia flowers. Plant Physiol 130:2142–2151
71. Dhaubhadel S, Farhangkhoee M, Chapman R (2008) Identification and characterisation of isoflavonoid specific glycosyltransferase and malonyltransferase from soybean seeds. J Exp Botany 59:981–994
72. Suzuki H, Nishino T, Nakayama T (2007) cDNA cloning of a BAHD acyltransferase from soybean (Glycine max): isoflavone 7-O-glucoside-6"-O-malonyltransferase. Phytochemistry 68:2035–2042
73. Du L, Halkier BA (1998) Biosynthesis of glucosinolates in the developing silique walls. Phytochemistry 48:1145–1150
74. Gijzen M, McGregor I, Seguin-Swart G (1989) Glucosinolate uptake by developing rapeseed embryos. Plant Physiol 89:260–263
75. Shitan N, Morita M, Yazaki K (2009) Identification of a nicotine transporter in leaf vacuoles of *Nicotiana tabacum*. Plant Signal Behav 4:530–532
76. Dhaubhadel S (2011) Regulation of isoflavonoid biosynthesis in soybean seeds. In: Ng T-B (ed) Soybean–biochemistry, chemistry and physiology. InTech, Croatia, pp 243–258
77. Aussenac T, Lacombe S, Dayde J (1998) Quantification of isoflavones by capillary zone electrophoresis in soybean seeds: effects of variety and environment. Am J Clin Nutr 68:1480S
78. Berger M, Rasolohery CA, Cazalis R, Daydé J (2008) Isoflavone accumulation kinetics in soybean seed cotyledons and hypocotyls: distinct pathways and genetic controls. Crop Sci 48:700–708
79. Hoeck JA, Fehr WR, Murphy PA, Welke GA (2000) Influence of genotype and environment on isoflavone contents of soybean. Crop Sci 40:48–51
80. Lee SJ, Yan W, Ahn JK, Chung IM (2003) Effects of year, site, genotype and their interactions on various soybean isoflavones. Field Crops Res 81:181–192
81. Hrazdina G, Wagner GJ (1985) Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. Arch Biochem Biophys 237:88–100

82. Eldridge AC, Kwolek WF (1983) Soybean isoflavones: effect of environment and variety on composition. J Agric Food Chem 31:394–396
83. Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot 62:2465–2483
84. Broun P (2005) Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. Curr Opin Plant Biol 8:272–279
85. Yi J, Derynck MR, Li X, Telmer P, Marsolais F, Dhaubhadel S (2010) A single repeat MYB transcription factor, GmMYB176, regulates CHS*8* gene expression and affects isoflavonoid biosynthesis in soybean. Plant J 62:1019–1034
86. Dhaubhadel S, Li X (2010) A new client for 14-3-3 proteins: GmMYB176, an R1 MYB transcription factor. Plant Signal Behav 5:1–3
87. Bai MY, Zhang LY, Gampala SS, Zhu SW, Song WY, Chong K, Wang ZY (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. Proc Natl Acad Sci USA 104:13839–13844
88. Paul AL, Folta KM, Ferl RJ (2008) 14-3-3 proteins, red light and photoperiodic flowering. Plant Signal Behav 3:511–515
89. Schoonheim PJ, Costa Pereira DD, De Boer AH (2009) Dual role for 14-3-3 proteins and ABF transcription factors in gibberellic acid and abscisic acid signalling in barley (Hordeum vulgare) aleurone cells. Plant Cell Environ 32:439–447
90. Sehnke PC, DeLille JM, Ferl RJ (2002) Consummating signal transduction: the role of 14-3-3 proteins in the completion of signal-induced transitions in protein activity. Plant Cell 14 Suppl:S339–354
91. Xu WF, Shi WM (2006) Expression profiling of the 14-3-3 gene family in response to salt stress and potassium and iron deficiencies in young tomato (*Solanum lycopersicum*) roots: analysis by real-time RT-PCR. Ann Bot 98:965–974
92. Muslin AJ, Tanner JW, Allen PM (1996) Interaction of 14-3-3 with signalling proteins is mediated by the recognition of phosphoserine. Cell 84:889–897
93. Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC (1997) The structural basis for 14-3-3: phosphopeptide binding specificity. Cell 91:961–971
94. Aitken A (2006) 14-3-3 proteins: a historic overview. Sem Cancer Biol 16:162–172
95. Fu H, Subramanian RR, Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 40:617–647
96. Li X, Dhaubhadel S (2011) Soybean 14-3-3 gene family: identification and molecular characterization. Planta 233:569–582
97. Li X, Chen L, Dhaubhadel S (2012) 14-3-3 proteins regulate the intracellular localization of the transcriptional activator GmMYB176 and affect isoflavonoid synthesis in soybean. Plant J 71:239–250
98. Britz SJ, Schomburg CJ, Kenworthy WJ (2010) Isoflavones in seeds of field-grown soybean: variation among genetic lines and environmental effects. J Am Oil Chem Soc 88:827–832
99. Gutierrez-Gonzalez J, Wu X, Gillman J, Lee J-D, Zhong R, Yu O, Shannon G, Ellersieck M, Nguyen H, Sleper D (2010) Intricate environment-modulated genetic networks control isoflavone accumulation in soybean seeds. BMC Plant Biol 10:105
100. Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K (1995) Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem 43:1184–1192
101. Vyn TJ, Yin XH, Bruulsema TW, Jackson CJC, Rajcan I, Brouder SM (2002) Potassium fertilization effects on isoflavone concentrations in soybean [*Glycine max* (L.) Merr.]. J Agric Food Chem 50:3501–3506
102. Gutierrez-Gonzalez JJ, Guttikonda S, Aldrich DL, Tran LSP, Zhong R, Yu O, Nguyen HT, Sleper DA (2010) Differential expression of isoflavone biosynthetic genes in soybean during water deficits. Plant Cell Physiol 51:936–948

103. Kirakosyan A, Kaufman P, Nelson RL, Kasperbauer MJ, Duke JA, Seymour E, Chang SC, Warber S, Bolling S (2005) Isoflavone levels in five soybean (*Glycine max*) genotypes are altered by phytochrome-mediated light treatments. J Agric Food Chem 54:54–58
104. Kim S-H, Jung W-S, Ahn J-K, Kim J-A, Chung I-M (2005) Quantitative analysis of the isoflavone content and biological growth of soybean (*Glycine max* L.) at elevated temperature, CO2 level and N application. J Sci Food Agric 85:2557–2566
105. Kitamura K, Igita K, Kikuchi A, Kudou S, Okubo K (1991) Low isoflavone content in some early maturing cultivars, so-called summer-type soybeans (*Glycine max* (L.) Merrill). Jpn J Breed 41:651–654
106. Lozovaya VV, Lygin AV, Ulanov AV, Nelson RL, Daydé J, Widholm JM (2005) Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop Sci 45:1934–1940

Chapter 2
Biosynthesis of Natural Products in Plants by Fungal Endophytes with an Emphasis on Swainsonine

Daniel Cook, Dale R. Gardner, James A. Pfister and Daniel Grum

Abstract Plant natural products are frequently used as chemotaxonomic markers which are indicative of select members of a family, genus, and/or species. However, the erratic occurrence of some natural products raises questions about their biosynthetic origin and significance as chemotaxonomic markers. Recent research has shown that fungal endophytes associated with plants are a rich source of natural products. The objective of this review is to highlight natural products found in plants that are reported to be derived from fungal endophytes and, when appropriate, briefly comment on the plant-endophyte interaction. We will summarize current knowledge on alkaloids synthesized by Clavicipitaceae endophytes, then on other diverse secondary metabolites including taxol and camptothecin. Specifically, we will highlight the role that fungal endophytes play in the synthesis of the indolizidine alkaloid swainsonine and the interaction between host and endophyte.

Keywords Ergot alkaloids · Swainsonine · Taxol · Endophytes · Clavicipitaceae · Undifilum

2.1 Introduction

Plant natural products are frequently used as chemotaxonomic markers which are indicative of select members of a family, genus, and/or species. However, the erratic occurrence of some natural products raises questions about their biosynthetic origin and significance as chemotaxonomic markers. Four mechanisms could explain the sporadic occurrence of natural products in unrelated taxa [1]: First, plant taxa may have lost their ability to produce the natural product; second, the biosynthetic pathways of a natural product may have originated multiple times over evolutionary history; third, the genes responsible for the biosynthesis of a natural product may have

D. Cook (✉) · D. R. Gardner · J. A. Pfister · D. Grum
Poisonous Plant Research Laboratory, Agricultural Research Service,
United States Department of Agriculture, 1150 E 1400 N,
Logan, UT 84341, United States
e-mail: Daniel.Cook@ARS.USDA.GOV

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application*,
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_2

been horizontally transferred between unrelated taxa [2]; or fourth, the natural product may be produced by a microbe associated with several, unrelated plant species.

Fungal endophytes are microbes that live within plants for part of their life cycle without causing any apparent disease symptoms [3]. Endophytes differ in their host range, tissues colonized, *in planta* biodiversity, *in planta* colonization, mode of transmission, and fitness benefit conferred to the host [4]. These criteria have been used to separate endophytes into different classes. For a more detailed review of the proposed classes of endophytes and their diagnostic criteria see Rodriguez et al. [4].

Recent research has shown that fungal endophytes associated with plants are a rich source of natural products [4, 5]. The objective of this review is to highlight natural products found in plants that are reported to be derived from fungal endophytes and, when appropriate, briefly comment on the plant-endophyte interaction. We will first summarize current knowledge on alkaloids synthesized by Clavicipitaceae endophytes (Sect. 2.2), then on diverse other secondary metabolites (Sect. 2.3). Specifically, we will highlight the role that fungal endophytes play in the synthesis of the indolizidine alkaloid swainsonine and the interaction between host and endophyte (Sect. 2.4). The review will not highlight plant mycorrhizal relationships.

2.2 Alkaloids Derived from Clavicipitaceous Endophytes

The most studied fungal endophyte system is the symbiotic association of the fungal endophytes in the genera *Epichloë* and *Neotyphodium* (Clavicipitaceae) with many cool season grasses (Poaceae). *Neotyphodium* species are asexual and grow within the intercellular spaces of their grass hosts, while *Epichloë* species represent the sexual states of several *Neotyphodium* species. *Neotyphodium* species are strictly vertically transmitted while *Epichloë* species are horizontally and vertically transmitted. *Epichloë* species are distinguished from *Neotyphodium* species because they are capable of exiting their plant hosts via the formation of sexual reproductive stroma on plant inflorescences.

Epichloë and *Neotyphodium* species may produce four classes of bioactive metabolites in their symbiotic associations with plants: ergot alkaloids, indole diterpenes, loline alkaloids, and peramine (Fig. 2.1). These four classes of alkaloids are derived from amino acid precursors, and the pathways are independent of one another. No individual fungal endophyte has been reported to produce representatives of all four classes; most produce metabolites belonging to one to three of the chemical classes [5].

The diverse suite of metabolites in the ergot alkaloid family can be grouped as clavines, simple amides of lysergic acid, or ergopeptines with the classification based on the structural complexity and position in the pathway [6, 7]. Like the ergot alkaloids, the indole-diterpenes represent a suite of products including the terpendoles, lolitrems, and janthitrems derived from oxidation and prenylation of a shared biosynthetic precursor, terpendole I [8, 9]. The lolines represent a family of aminopyrrolizidine alkaloids, derived from homoserine and proline. Lastly,

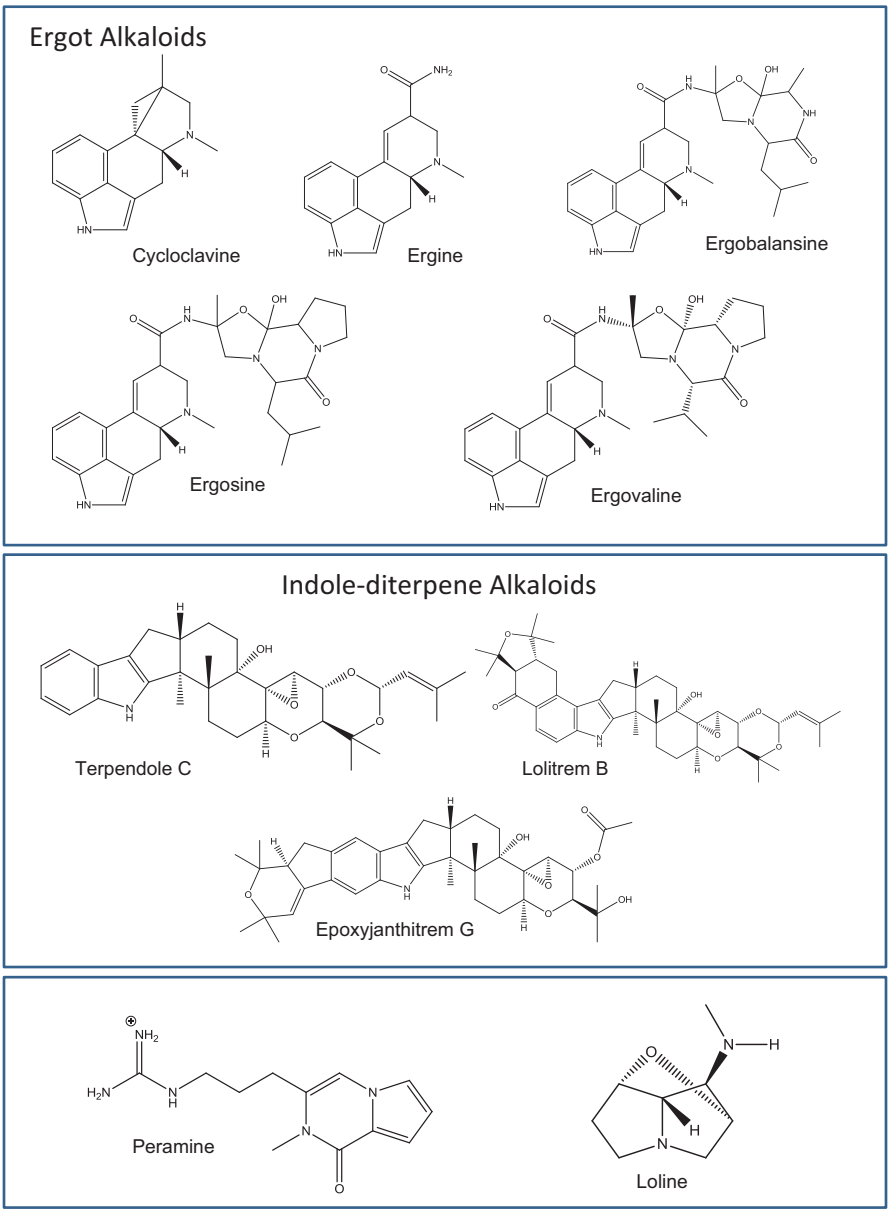


Fig. 2.1 Chemical structures of representative alkaloids produced by Clavicipitaceae endophytes associated with the grasses and morning glory families

peramine represents a single alkaloid rather than a family of alkaloids and is derived from a dipeptide possibly made up of arginine and a precursor to proline. *Epichloë* and *Neotyphodium* endophytes of grasses and the alkaloids associated with this

interaction affect herbivores and significantly impact ecological communities. For a more detailed commentary on each of these alkaloids and their biology and the effects of this symbiotic interaction, readers are referred to Schardl et al. [5, 10].

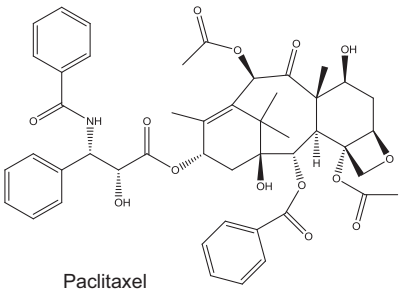
Not only are ergot alkaloids found in cool season grasses, but they are also present in select taxa of the Convolvulaceae, the morning glory family [11]. The three major types of ergot alkaloids have been detected in the Convolvulaceae [11], as well as the lolines in one taxon of the Convolvulaceae, *Argyreia mollis* [12]. In regard to the other bioactive metabolites found in grasses, there are no published reports of any Convolvulaceae taxa containing the indole diterpenes or peramine. However, there are a number of reports of livestock having a tremorgenic syndrome caused by feeding on select *Ipomoea* species including *Ipomoea muelleri* and *Ipomoea asarifolia* [13, 14]. These cases suggest that *Ipomoea* species may contain the indole diterpenes, as they are known to be tremoregenic, but this remains to be verified. Recent research has demonstrated the presence of clavicipitaceous epibiotic fungal symbionts described as *Periglandula* species in *Ipomoea* taxa [1, 15–17]. These fungi are vertically transmitted and appear to have a narrow host range, as the described species to date occur on different plant hosts. *Periglandula* species are the only clavicipitaceous fungi known to be associated with a dicot host [15]. The ecological effects of this interaction have yet to be studied; however, effects are likely to be substantial due to the bioactivities of the endophyte-derived alkaloids.

2.3 Other Natural Products

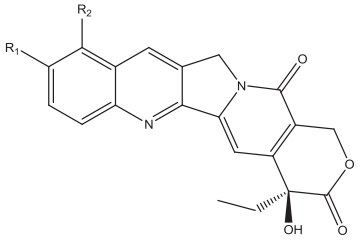
Numerous other natural products in plants are reported to be produced by fungal endophytes associated with the host. Some examples include paclitaxel (also known as taxol), podophyllotoxin, deoxypodophyllotoxin, camptothecin and structural analogues, hypericin and emodin, and azadirachtin (Fig. 2.2). In each of these cases, the compounds are thought to be produced by the plant as well as an endophyte that has been isolated from the plant host. Furthermore, in the cases of paclitaxel and camptothecin, the biosynthetic pathways *in planta* have been described, and several enzymes in the pathways have been characterized.

2.3.1 Taxol

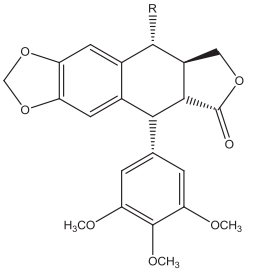
Taxol, a taxane-type diterpene, is a tetracyclic lactam found in the genus *Taxus* [18]. All *Taxus* species are reported to contain taxanes. Taxol is an anticancer drug that acts as a microtubule inhibitor. Taxol was first reported to be produced by *Taxomyces andreanae*, an endophytic fungus associated with *Taxus brevifolia* [19]. Since then, more than 20 genera that produce taxol have been isolated, representing several species of unrelated endophytic fungi [20]. In several cases, more than one endophytic isolate from the same host has been reported to produce taxol. In one study of 109 endophytic isolates cultured from *Taxus chinensis*, 28 isolates



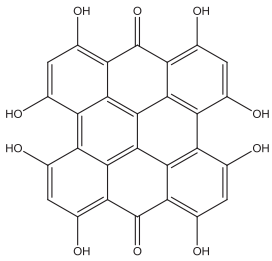
Paclitaxel



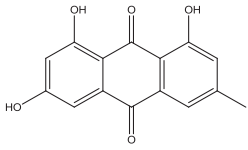
Camptothecin R = H; R_1 = H
9-Methoxycamptothecin R = OCH_3; R_1 = H
10-Hydroxycamptothecin R = H; R_1 = OH



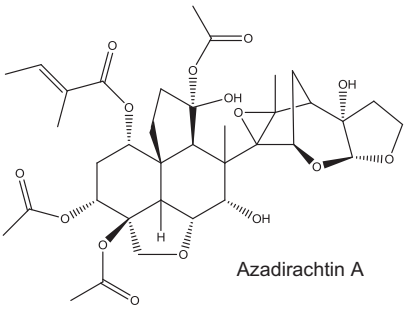
Podophyllotoxin R = OH
Deoxypodophyllotoxin R = H



Hypericin



Emodin



Azadirachtin A

Fig. 2.2 Chemical structures of other highlighted natural products reported to be produced by endophytes

were reported to produce taxol [21]. Taxol-producing endophytes have been isolated from various hosts including *Taxus* species, cypress [22], *Wollemia* pine [23], and a variety of other non-*Taxus* species [24, 25]. These hosts include some that are reported to contain taxol and some that are not. None of the taxol-producing endophytes has been reported to contain any intermediates that are detected *in planta* for the taxol biosynthetic pathway. Furthermore, there are no reports of plant taxol biosynthetic enzymes found in fungi. In the cases where the endophytic isolates

were isolated from *Taxus* species, the authors propose that horizontal gene transfer was responsible for the biosynthetic machinery of the plant being transferred to the fungus, although no data are presented in support of this hypothesis. In cases where taxol-producing endophytic isolates were isolated from hosts that are reported not to produce taxol, the authors suggest that the endophytic isolate may have been associated with a *Taxus* species, but through time came to be associated with a different host [23].

2.3.2 Podophyllotoxin

Podophyllotoxin, podophyllotoxin glycoside, deoxypodophyllotoxin, and related analogues are present in four plant families: the Berberidaceae, Cupressaceae, Polygalaceae, and Linaceae [26]. These compounds are aryl tetralin lignans, and have various bioactivities including but not limited to antiviral [27], anticancer [28], antiproliferative [29], and insecticidal [30] properties. In fact, podophyllotoxin is in great demand as a precursor in the synthesis of topoisomerase inhibitors that are anticancer drugs. Recently, endophytes have been isolated from both *Podophyllum* and *Juniperus* species that are reported to produce these structurally related compounds [31–33]. In two *Podophyllum* species, two unrelated endophytes were identified: in *Podophyllum hexandrum* the endophyte *Trametes hirsuta* was identified that produces podophyllotoxin, podophyllotoxin glycoside, and deoxypodophyllotoxin [32], whereas in *Podophyllum peltatum* the endophyte *Phialocephala fortinii* was identified that produces podophyllotoxin [31]. In *Juniperus communis*, the endophyte *Aspergillus fumigatus* was identified and reported to produce deoxypodophyllotoxin [33]. Interestingly, in the latter two examples the authors do not mention if the endophyte produces any of the other structurally related analogues. In all three cases, the authors propose that horizontal gene transfer was responsible for the biosynthetic machinery from the plant being transferred to the fungus, although no data are presented in support of this hypothesis.

2.3.3 Hypericin

Hypericin is a naphtodianthrone derivative of significant medicinal value isolated from St. John's Wort, *Hypericum perforatum* [34]. Hypericin is reported to have a number of bioactivities including but not limited to being an antidepressant [35], anti-inflammatory, and antiviral [36]. Hypericin is a photodynamic compound activated by light, and animals consuming St. John's Wort can suffer from primary photosensitization due to hypericin. Emodin is thought to be a biosynthetic precursor of hypericin [36, 37]. Recently, an endophyte isolated from the inner stem tissues of *Hypericum perforatum* and found to produce both emodin and hypericin was identified as *Thielavia subthermophila* [37].

2.3.4 Camptothecin

Camptothecin, a pentacyclic quinoline alkaloid, has been reported from several genera in unrelated angiosperm families [38]. For example, camptothecin has been reported in *Camptotheca* spp. (Nyssaceae) [39], *Ophiorrhiza* spp. (Rubiaceae) [40], *Nothapodytes foetida*, and *Apodytes dimidiate* (Icacinaceae) [41], and two genera of the Apocynaceae and Gelsemiaceae [42, 43]. Camptothecin and its structural analogues 9-methoxycamptothecin and 10-hydroxycamptothecin are potent neoplastic compounds that are important because they inhibit the enzyme topoisomerase I which is required for DNA replication and transcription [38]. In fact, two semisynthetic drugs targeting different cancers are derived from camptothecin [38]. Endophytes isolated from *Nothapodytes foetida*, *Apodytes dimidiate*, and *Camptotheca acuminata* have been reported to produce camptothecin [44–47]. Two unrelated endophytes have been isolated from *Nothapodytes foetida* that produce camptothecin [44, 45]. Interestingly, separate strains of *Fusarium solani* that produce camptothecin were isolated from two unrelated plants (*Apodytes dimidiate* and *Camptotheca acuminata*) [46, 47]. In two of the above examples, the isolated endophytes produce camptothecin and its structural analogues [46, 47] while in the other two examples camptothecin is produced, but the authors do not mention if the endophyte produces other structurally related compounds [44, 45].

The endophyte isolated from *Camptotheca acuminata* was particularly intriguing because it lost its ability to produce camptothecin at the third subculture and continued as such to the seventh generation [46]. To further investigate the loss of camptothecin synthesis in the endophyte isolated from *Camptotheca acuminata*, the authors took advantage of the fact that the biosynthetic pathway of camptothecin is known and several enzymes in the pathway have been described [48]. The authors identified geraniol 10-hydroxylase, secologanin synthase, and tryptophan decarboxylase in the *Fusarium solani* strain isolated from *Camptotheca acuminata* [48], which were 100 % identical to the host enzymes. The authors found no evidence of the strictosidine synthase in the endophyte, the enzyme in the biosynthethic pathway of camptothecin that forms strictosidine from tryptamine and secologanin. The authors proposed a cross-species biosynthetic pathway where the endophyte uses the host strictosidine synthase. Furthermore, the authors showed that the genes involved in camptothecin biosynthesis in the endophyte had undergone random nonsynonymous mutations from generation 1 to generation 7 to further explain the inability of the endophyte to produce camptothecin and its precursors [48].

2.3.5 Azadirachtin

Azadirachtin A and its structural analogues are highly oxygenated tetranortriterpenoids isolated from the Indian neem or Indian lilac, *Azadirachta indica* [49]. These compounds are reported to have various bioactivities, including as insecticides that act as antifeedants and have growth-regulating properties [50]. They are found in

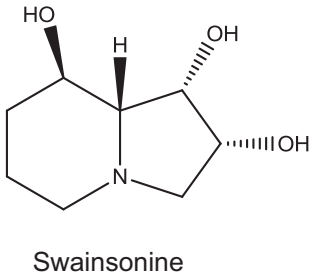


Fig. 2.3 Chemical structure of swainsonine

all parts of the plant, with the highest concentrations in the seeds [49]. Recently, an endophytic fungus identified as *Eupenicillium parvum* was isolated from the plant host, *Azadirachta indica*, that was capable of producing azadirachtin A and B but not the other structural analogues [51].

2.4 Swainsonine

Several species in the legume (Fabaceae) genera *Astragalus*, *Oxytropis*, and *Swainsona* are toxic to grazing livestock in the Americas, Asia, and Australia [52–55]. Locoism, a disease induced by prolonged grazing of some *Astragalus* and *Oxytropis* species generally called locoweeds, was first noted by the Spanish conquistadors, and again during the settlement of western North America by pioneers [52, 53, 56]. Clinical signs and pathology of locoism, a neurologic disease, are similar in animals intoxicated by locoweed species and *Swainsona* species [57, 58]. Swainsonine (Fig. 2.3), a trihydroxyindolizidine alkaloid, was first identified as the bioactive principle of the neurologic disease in *Swainsona canescens*, a legume native to Australia [59], and subsequently identified as the active principle in locoweeds [60]. Swainsonine inhibits the enzymes α-mannosidase and mannosidase II, resulting in lysosomal storage disease and altered glycoprotein synthesis [61, 62].

A fungal endophyte, *Undifilum oxytropis*, previously described as an *Embellesia* species [63], is reported to produce swainsonine in locoweeds [64, 65]. The *Undifilum* genus (Pleosporaceae) is related to the genera *Alternaria*, *Embellesia*, and *Ulocladium* [65]. In general, *Undifilum* species are associated with swainsonine-containing *Astragalus* and *Oxytropis* species in North America and China [65–67]. Other species of *Undifilum* have been found and characterized in the swainsonine-containing plant species *Astragalus lentiginosus* and *Astragalus mollissimus* [67]. *Undifilum bornmuelleri*, a pathogen of the legume *Securigera varia* [65], is reported to not produce swainsonine. *Undifilum* species associated with locoweeds are vertically transmitted and have no apparent sexual stage [68]. *Undifilum* species appear to have a narrow host range, as different plant species are associated with unique *Undifilum* species [65, 67].

Swainsonine concentrations vary greatly among plant species, varieties and populations, often ranging from not being detected to greater than 0.2 %. For example, *Astragalus* locoweed species generally have greater swainsonine concentrations than *Oxytropis* locoweed species in North America, although this depends on the specific taxa being compared [69]. Swainsonine concentrations vary greatly among different varieties of some *Astragalus* and *Oxytropis* species. For example, some *Oxytropis lambertii* and *Astragalus mollissimus* varieties contain swainsonine, while others contain very little or no swainsonine [69, 70]. Additionally, swainsonine concentrations vary greatly among populations, for example some populations of *O. lambertii* var. *bigelovii* contain swainsonine while others contain very little or no swainsonine. Furthermore, within toxic populations of locoweeds, swainsonine concentrations vary greatly among individual plants.

Initial reports demonstrated that the endophyte usually could be cultured only from locoweed samples, with swainsonine concentrations greater than 0.01 %, but could be detected by polymerase chain reaction (PCR) in all samples regardless of swainsonine concentration [69]. To further describe the plant–endophyte–swainsonine relationship and to investigate the differences in swainsonine concentrations among individual plants in locoweed populations, a method was developed to quantitate the endophyte in plant samples. Using quantitative polymerase chain reaction (qPCR), the amount of endophyte was measured in the *Astragalus* and *Oxytropis* locoweeds with different swainsonine concentrations. Two chemotypes of plants were identified, namely chemotype 1 plants, which contain swainsonine concentrations greater than 0.01 %, and chemotype 2 plants, which have concentrations below 0.01 % (generally near 0.001 % or not detected) (Fig. 2.4a) [71, 72]. These two chemotypes were determined to differ significantly in the amount of endophyte they contain, which may help to explain the difference in swainsonine concentrations of the respective host plants (Fig. 2.4b) [71, 72].

Swainsonine and endophyte concentrations have been shown to be influenced by the plant part [71, 72] and phenological stage of the plant [73, 74]. Swainsonine was found in all plant parts, with greater concentrations in aboveground parts than in below-ground parts (Fig. 2.4a); the endophyte *Undifilum* was also found in all plant parts, again with greater concentrations in aboveground parts than in the root (Fig. 2.4b) [71, 72]. The root crown has endophyte amounts equivalent to aboveground parts, which we believe may serve as a reservoir for the endophyte for the following year's growth, as many locoweeds are perennial plants [71, 72]. Swainsonine concentrations are greatest in floral parts and seeds [73]. This is consistent with the optimal defense theory of protecting reproductively valuable plant and endophyte tissues. Swainsonine and endophyte amounts in aboveground parts increase throughout the growing season until the plant reaches maturity [73, 74]. As plants senesce, swainsonine concentrations decrease significantly [73]. Swainsonine and endophyte amounts are highly correlated over the growing season, suggesting the endophyte amount plays a critical role in determining swainsonine concentration in the plant [73].

Three mechanisms were proposed that may explain the origin of chemotype 2 plants in *Astragalus* and *Oxytropis* locoweeds [72]. First, different endophyte

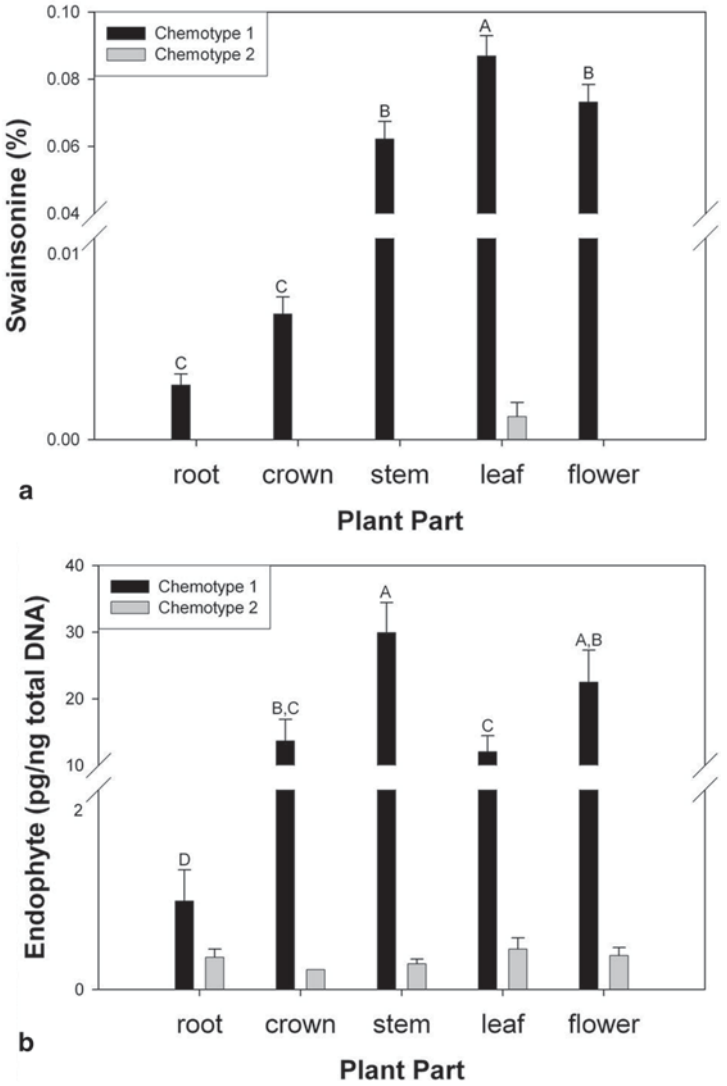


Fig. 2.4 Comparison of swainsonine and endophyte concentrations among plant parts and between two chemotypes of *Oxytropis sericea*. Mean swainsonine (%) and endophyte (pg/ng total DNA) concentrations (±SE) **a** from different plant parts (root, crown, scape, leaf, and flower/pod) and **b** from two chemotypes of *O. sericea* plants [71]

genotypes distributed across host plants may account for differences in swainsonine accumulation. However, recent work does not support this suggestion, as we have reported that the internal transcribed spacer (ITS) of the rDNA region of *Undifilum* spp. from three locoweed species are identical between chemotype 1 and 2 plants [72].

Second, plants with no detectable or very low swainsonine concentrations may have arisen, due to imperfect transmission of some critical amount of the endophyte to the seed or seedling [72, 75] that is required for new plants to be colonized and produce concentrations of swainsonine characteristic of chemotype 1 plants. To test this, the transmission of the endophyte in these two chemotypes was investigated. Seeds derived from chemotype 1 and chemotype 2 plants were germinated and the chemical phenotype of the resulting plants was evaluated. Progeny derived from the seeds of chemotype 1 plants had swainsonine concentrations consistent with both chemotype 1 and chemotype 2 plants, demonstrating imperfect transmission. All progeny derived from seeds of a chemotype 2 plant had swainsonine concentrations consistent with chemotype 2 plants [76], suggesting that these seeds may lack some critical amount of endophyte.

Third, low swainsonine chemotype 2 plants may suppress endophyte growth and thus alkaloid production due to a plant genotype by endophyte interaction similar to that observed in grasses [72, 77, 78]. To investigate this possibility further, swainsonine and endophyte amounts were investigated in seeds derived from chemotype 1 and chemotype 2 plants. Seeds derived from a chemotype 1 plant had greater swainsonine and endophyte amounts than seeds derived from a chemotype 2 plant. We hypothesized that seed endophyte amounts were a critical factor in determining the swainsonine concentration in the resulting plant. To address this hypothesis, we investigated if plants derived from chemotype 1 seeds could have swainsonine concentrations like chemotype 2 plants by reducing the amount of *Undifilum* in chemotype 1 seeds [79]. Furthermore, we investigated if plants derived from chemotype 2 seeds could have swainsonine concentrations like chemotype 1 plants by inoculating chemotype 2 embryos with *Undifilum* [79]. Plants derived from chemotype 1 seeds, where the amount of endophyte was reduced by seed-coat removal and fungicide treatment, had swainsonine and endophyte amounts consistent with chemotype 2 plants (Fig. 2.5). On the other hand, plants derived from *Undifilum*-inoculated chemotype 1 and chemotype 2 embryos had swainsonine and endophyte amounts consistent with chemotype 1 plants (Fig. 2.5). Similar results were seen for *A. lentiginosus* and *A. mollissimus*. This suggests that endophyte amount at seed germination is a determining factor if a plant exhibits a swainsonine chemotype 1 or 2. If the plant endophyte by genotype interaction is a key determinant, one may expect that plants derived from inoculated chemotype 2 embryo would have swainsonine and endophyte amounts similar to a chemotype 2 plant.

Concentrations of swainsonine are reported to differ among populations of *Oxytropis sericea*. For example, mean concentrations were approximately three times greater in plants from Cuchara, CO (0.40%) than plants from Park Valley, UT (0.15%) [80]. Differences in concentrations among populations may be due to the environment, genotype of the plant, and/or endophyte, or some combination of these factors. To identify the contributing factor(s) responsible for the observed swainsonine differences, plants were grown in a common garden. Swainsonine concentrations were different between the two populations in plants grown in a common garden [80]. The endophyte cultured from each population produced different amounts of swainsonine in vitro when grown under common culture conditions

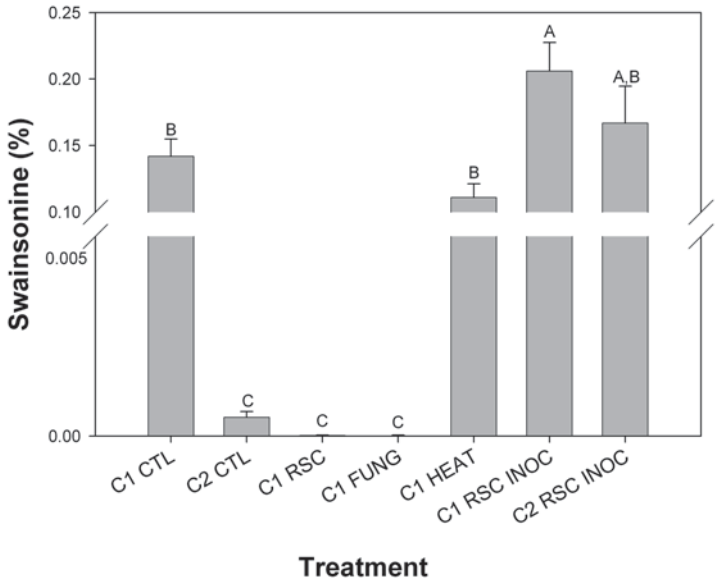


Fig. 2.5 Swainsonine concentrations in *Oxytropis sericea* across different seed treatments. Mean swainsonine concentrations (%, ± SE) from plants grown after each seed treatment: *C1 CTL* (plants derived from control chemotype 1 seeds), *C2 CTL* (plants derived from control chemotype 2 seeds), *C1 RSC* (plants derived from chemotype 1 seeds where the seed coat was removed), *C1 FUNG* (plants derived from chemotype 1 fungicide-treated seeds), *C1 HEAT* (plants derived from chemotype 1 heat-treated seeds), *C1 RSC INOC* (plants derived from endophyte-inoculated chemotype 1 embryos), and *C2 RSC INOC* (plants derived from endophyte-inoculated chemotype 2 embryos). *Y axis* shows a break from 0.005 % swainsonine to 0.1 %. Different *letters* above each *bar* represent significant differences at $P < 0.05$ [77]

[80]. Furthermore, a cross-inoculation experiment was performed [80], wherein the endophyte from one population was inoculated to the embryo from the other location, and vice versa. Results from this experiment and others clearly demonstrated that the endophyte amount was responsible for differences in swainsonine concentration between the two populations, not plant genotype or differences in endophyte amount.

Limited studies exist regarding the ecological benefit or role of swainsonine and the influence of environmental conditions on swainsonine production. Swainsonine does not deter grazing; in fact, animals take 2–3 weeks to show clinical signs and continue grazing locoweeds after becoming intoxicated [81]. Furthermore, swainsonine concentrations are reported not to change in response to the clipping used to simulate herbivory [82]. Swainsonine has not been reported to be bioactive against insects, fungi, or bacteria; one preliminary study suggested that it has no effect on a native weevil herbivore of locoweeds [83]. Legumes are known for forming symbioses with nitrogen-fixing bacteria, and investigators found that swainsonine concentrations were greater in plants inoculated with one strain of *Rhizobium* but not others, which suggested an interaction between the two classes of symbionts [84].

An alternative interpretation is that the host may have increased substrate availability for swainsonine production due to the improved nitrogen status. However, another report showed no consistent differences in swainsonine concentrations across locoweed taxa with deficient or adequate nitrogen, when nitrogen was supplied through fertilizer [85]. Lastly, water stress was shown to result in increased swainsonine concentrations in some locoweed species, but not others [86]. Thus, the ecological role of swainsonine for the endophyte and/or plant remains unclear, and swainsonine production does not appear to respond in a consistent manner to variation in environmental conditions experienced by the plant.

The following observations support that swainsonine is a fungal-endophyte-derived secondary metabolite in locoweeds, and is not produced by the host plant: (1) *Astragalus* and *Oxytropis* species that contain swainsonine are infected with *Undifilum* species; (2) plants derived from *Astragalus* and *Oxytropis* embryos where the seed coat was removed have no detectable swainsonine, or have concentrations less than 0.001 % [68, 79]; (3) plants derived from fungicide-treated *Astragalus* and *Oxytropis* seeds have no detectable swainsonine or have concentrations less than 0.001 % [79]; (4) *Undifilum* species isolated from locoweeds produce swainsonine in vitro [64]; (5) plants derived from *Undifilum*-inoculated embryos have swainsonine concentrations greater than 0.01 % [79]; and (6) rats fed *U. oxytropis* developed lesions and clinical signs similar to those fed swainsonine-containing *O. lambertii* [87].

In addition to the *Astragalus* and *Oxytropis* species, swainsonine has been reported in the Fabaceae genus *Swainsona* and two other plant families, the Convolvulaceae and the Malvaceae. As mentioned previously, swainsonine was first isolated from *S. canescens*, and some other *Swainsona* species including *Swainsona galegifolia* and *Swainsona greyana* contain swainsonine [88]. In the Convolvulaceae, some *Ipomoea* and *Turbina* species, including *Ipomoea carnea*, *Ipomoea riedelli*, *Ipomoea sericophylla*, and *Turbina cordata* have been reported to contain swainsonine [89–91]. Only a single species of Malvaceae, *Sida carpinifolia*, has been reported to contain swainsonine [92]. Like the legumes, swainsonine was identified in the plant species associated with these families due to livestock poisoning and subsequent economic impact. The presence of swainsonine in these species may be a case of convergent evolution or horizontal gene transfer; however, due to the sporadic occurrence of swainsonine in these genera, it seems likely that a swainsonine-producing fungal endosymbiont is associated with these taxa that contain swainsonine.

Swainsonine is reported to be produced by *Undifilum* species (Pleosporales) associated with locoweeds, and additionally by two phylogenetically disjunct fungi not associated with plants as endophytes. *Rhizoctonia leguminicola* (Cantharellales) is a fungal pathogen of red clover (*Trifolium pratense*) that causes black patch disease in the plant, and *Metarhizium anisopliae* is an entomopathogen that attaches to the outside of an insect, grows internally, and causes death (Hypocreales) [93, 94]. As a result, we hypothesized that a fungal endosymbiont may be associated with swainsonine-containing *Swainsona* and *Ipomoea* species. Furthermore, we speculated that it may be vertically transmitted and thus associated with seeds. To investigate this possibility, seeds from *S. canescens* and *I. carnea* were surface

sterilized and placed on media to culture any seed-associated endophytes. Both plant species yielded a single endosymbiont that produced swainsonine in vitro. Each endosymbiont was detected by PCR and recovered by culturing from swainsonine-containing plant material from each respective species. Lastly, preliminary data suggest that these endophytes are vertically transmitted and have a narrow host range. The endosymbiont from *S. canescens* was characterized by morphologic and phylogenetic methods and represents a novel species of *Undifilum*. The endosymbiont from *I. carnea* belongs to the Ascomycete order Chaetothyriales and is not phylogenetically similar to any described fungi [95]. Furthermore, *I. carnea* plants derived from fungicide-treated seeds lack swainsonine, further demonstrating that the endosymbiont, but not the host plant, produces swainsonine [95]. These studies [95] provide more evidence that the presence of swainsonine in plants is associated with the presence of a vertically transmitted endosymbiont.

2.5 Summary

Recent research has increasingly shown that fungal endophytes associated with plants are a rich source of diverse natural products found in plants. The alkaloids, derived from the Clavicipitaceae endophyte association with grasses, and the morning glories represent one of the more well-studied examples of natural products derived from endophytes. The indolizidine alkaloid swainsonine and the association between swainsonine-containing plant hosts with the associated endophytic fungi represents a parallel example where only the endophyte, not the host plant, produces the alkaloid. One of the most striking differences between the two systems is that all vertically transmitted endophytic fungi that produce the ergot alkaloids, indole diterpenes, lolines, and peramine are derived from Clavicipitaceae, regardless of the plant family with which they are associated. In contrast, vertically transmitted endophytes that produce swainsonine are derived from different fungal orders that form relationships with specific plant families. In the light of this observation, the evolutionary history of the swainsonine biosynthetic pathway in these diverse fungi is particularly intriguing.

In regard to the other natural products discussed here, future work is needed to further describe the interaction between the plant hosts and fungal endophytes. In most of these examples, an endophyte was simply isolated from the plant host that produces the natural product of interest. Additionally, in most of these examples there was evidence that the plant host produced the natural product as well, so horizontal transfer of the biosynthetic pathway to the fungal endophyte was proposed. If these cases are verified as reported, this represents a tremendous example of horizontal gene transfer to various unrelated fungi in which all the biosynthetic genes are functionally transferred. These examples are thus fundamentally different from the Clavicipitaceae endophyte association and the *Undifilum* endophyte association wherein the endophytes found in related plant taxa are also related.

References

1. Kucht S, Groß J, Hussein Y, Grothe T, Keller U, Basar S, König WA, Steiner U, Leistner E (2004) Elimination of ergoline alkaloids following treatment of *pomoea asarifolia* (Convolvulaceae) with fungicides. Planta 219:619–625
2. Bushman F (2002) Lateral DNA transfer. Mechanisms and consequences. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
3. Bacon CW, White JF (2000) Microbial endophytes. Marcel Deker, NewYork
4. Rodriguez RJ, White JF Jr, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330
5. Schardl CL, Young CA, Faulkner JR, Florea S, Pan J (2012) Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecol 5:331–344
6. Lorenz N, Haarmann T, Pazoutov S, Jung M, Tudzynski P (2009) The ergot alkaloid gene cluster: functional analyses and evolutionary aspects. Phytochemistry 70:1822–1832
7. Panaccione DG (2010) Ergot alkaloids. In: Hofrichter M (ed) The Mycota, vol. X, industrial applications, 2nd edn. Springer, Berlin-Heidelburg, pp 195–214
8. Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B (2006) A complex gene cluster for indolediterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genet Biol 43:679–693
9. Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B (2009) Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltm* gene analysis. Appl Environ Microbiol 75:2200–2211
10. Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol 55:315–340
11. Eich E (2008) Solanaceae and Convolvulaceae: secondary metabolites: biosynthesis, chemotaxonomy, biological and economic significance. Tryptophan-derived alkaloids. Springer, Berlin, pp 213–259
12. Tofern B, Kaloga M, Witte L, Hartmann T, Eich E (1999) Phytochemistry and chemotaxonomy of the Convolvulaceae part 8—Occurrence of loline alkaloids in Argyreia mollis (Convolvulaceae). Phytochemistry 51:1177–1180
13. Gardiner MR, Royce R, Oldroyd B (1965) Ipomoea muelleri intoxication of sheep in western Australia. Brit Vet J 121:272–277
14. Araújo JAS, Riet-Correa F, Medeiros RMT, Soares MP, Oliveira DM, Carvalho FKL (2008) Intoxicação experimental por Ipomoea asarifolia (Convolvulaceae) em caprinos e ovinos. Pesquisa Vet Brasil 28:488–494
15. Steiner U, Ahimsa-Muller MA, Markert A, Kucht S, Gross J, Kauf N, Kuzma M, Zych M, Lamshöft M, Furmanowa M, Knoop V, Drewke C, Leistner E (2006) Molecular characterization of a seed transmitted clavicipitaceous fungus occurring on dicotyledoneous plants (Convolvulaceae). Planta 224:533–544
16. Steiner U, Hellwig S, Leistner E (2008) Specificity in the interaction between an epibiotic clavicipitalean fungus and its convolvulaceous host in a fungus/plant symbiotum. Plant Signal Behav 3:704–706
17. Steiner U, Leibner S, Schardl CL, Leuchtmann A, Leistner E (2011) Periglandula, a new fungal genus within the Clavicipitaceae and its association with Convolvulaceae. Mycologia 103:1133–1145
18. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 93:2325–2327
19. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Science 260:214–216
20. Zhou X, Zhu H, Liu L, Lin J, Tang K (2010) A review: recent advances and future prospects of taxol-producing endophyte fungi. Appl Microbiol Biotechnol 86:1707–1717

21. Zhu JX, Li YC, Meng L (2008) Comparative study on different parts of taxol-producing endophytic fungi from T. chinesis in Taihang Mountain. Biotechnol Bull 4:191–194
22. Yan B, Bi JN, Ji Y, Wang W, Zhao Y, Zhu X (2007) An endophytic taxol-producing fungus from cypress. Acta Sci Nat Univ Nankaiensis (Nat Sci Edi) 40:67–70
23. Strobel GA, Hess WM, Li JY (1997) Pestalotiopsis guepinii, a taxol producing endophyte of the Wollemi pine, Wollemia nobilis. Aust J Bot 45:1073–1082
24. Li JY, Sidhu RS, Ford EJ, Long DM, Hess WM, Strobel GA (1998) The induction of taxol production in the endophytic fungus Periconia sp. from Torreya grandifolia. J Ind Microbiol Biotechnol 20:259–264
25. Sun DF, Ran XQ, Wang JF (2008) Isolation and identification of a taxol-producing endophytic fungus from Podocrapus. Acta Microbiol Sin 48:589–595
26. Petersen M, Alfermann AW (2001) The production of cytotoxic lignans by plant cell cultures. Appl Microbiol Biotechnol 55:135–142
27. Sudo K, Konno K, Shigeta S, Yokota T (1998) Inhibitory effects of podophyllotoxin derivatives on Herpes simplex virus replication. Antivir Chem Chemother 9:263–267
28. Kim Y, Kim SB, You YJ, Ahn B-Z (2002) Deoxypodophyllotoxin; the cytotoxic and antiangiogenic component from Pulsatilla koreana. Planta Med 68:271–274
29. Ikeda R, Nagao T, Okabe H, Nakano Y, Matsunaga H, Katano M, Mori M (1998) Antiproliferative constituents in Umbelliferae plants. III. Constituents in the root and the ground part of Anthriscus sylvestris Hoffm. Chem Pharm Bull 46:871–874
30. Gao R, Gao CF, Tian X, Yu X, Di X, Xiao H, Zhang X (2004) Insecticidal activity of deoxypodophyllotoxin, isolated from Juniperus sabina L, and related lignans against larvae of Pieris rapae L. Pest Manage Sci 60:1131–1136
31. Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from Podophyllum peltatum produce podophyllotoxin. J Nat Prod 69:1121–1124
32. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, Amna T, Ahmed B, Verma V, Singh S, Sagar R, Sharma A, Kumar R, Sharma RK, Qazi GN (2006) The endophytic fungus Trametes hirsuta as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 122:494–510
33. Kusari S, Lamshöft M, Spiteller M (2009) Aspergillus fumigatus Fresenius, an endophytic fungus from Juniperus communis L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107:1019–1030
34. Nahrstedt A, Butterweck V (1997) Biologically active and other chemical constituents of the herb of Hypericum perforatum L. Pharmacopsychiatry 30:129–134
35. Tammaro F, Xepapadakis G (1986) Plants used in phytotherapy, cosmetics and dyeing in the Pramanda district (Epirus, North-West Greece). J Ethnopharmacol 16:167–174
36. Kubin A, Wierrani F, Burner U, Alth G, Grünberger W (2005) Hypericin–the facts about a controversial agent. Curr Pharm Des 11:233–253
37. Kusari S, Lamshöft M, Zühlke S, Spiteller M (2008) An endophytic fungus from Hypericum perforatum that produces hypericin. J Nat Prod 71:159–162
38. Lorence A, Nessler CL (2004) Camptothecin: over four decades of surprising findings. Phytochemistry 65:2731–2841
39. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA (1966) Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata. J Am Chem Soc 88:3888–3890
40. Saito K, Sudo H, Yamazaki M, Moseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2001) Feasible production of camptothecin by hairy root culture of Ophiorrhiza pumila. Plant Cell Rep 20:267–271
41. Aiyama R, Nagai H, Nokata K, Shinohara C, Sawada S (1988) A camptothecin derivative from Notapodytes foetida. Phytochemistry 27:3663–3664
42. Gunasekera SP, Badawi MM, Cordell GA, Farnsworth NR, Chitnis M (1979) Plant anticancer agents X. Isolation of camptothecin and 9-methoxycamptothecin from Ervatamia heyneana. J Nat Prod 42:475–477

43. Dai JR, Cardellina JH, Boyd MR (1999) 20-Ob-Glucopyranosyl camptothecin from Mostuea brunonis: a potential camptothecin pro-drug with improved solubility. J Nat Prod 62:1427–1429
44. Puri SC, Verma V, Amna T, Qazi GN, Spiteller M (2005) An endophytic fungus from Nothapodytes foetida that produces camptothecin. J Nat Prod 68:1717–1719
45. Rehman S Shawl AS, Kour A, Kour A, Athar M, Andrabi R, Sultan P, Qazi GN (2008) An endophytic Neurospora sp. from Nothapodytes foetida producing camptothecin. Appl Biochem Micro 44:203–209
46. Kusari S, Zühlke S, Spiteller M (2009) An endophytic fungus from Camptotheca acuminata that produces camptothecin and analogues. J Nat Prod 72:2–7
47. Shweta S, Zuehlke S, Ramesha BT, Priti V, Mohana Kumar P, Ravikanth G, Spiteller M, Vasudeva R, Uma Shaanker R (2010) Endophytic fungal strains of Fusarium solani, from Apodytes dimidiate E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry 71:117–122
48. Kusari S, Zühlke S, Spiteller M (2011) Effect of artificial reconstitution of the interaction between the plant Camptotheca acuminata and the fungal endophyte Fusarium solani on camptothecin biosynthesis. J Nat Prod 74:764–777
49. Butterworth JH (1968) Isolation of a substance that suppresses feeding in locusts. Chem Comm 23–24
50. Lay SV, Denholm AA, Wood A (1993) The chemistry of azadirachtin. Nat Prod Rep 10:109–157
51. Kusari S, Verma VC, Lamshöft M, Spiteller M (2012) An endophytic fungus from Azadirachta indica A. Juss. that produces azadirachtin. World J Microbiol Biotechnol 28:1287–1294
52. Marsh CD (1909) The locoweed disease of the plains. United States Department of Agriculture Bureau Animal Industry Bulletin, No. 112
53. Marsh CD, Clawson AB (1936) The locoweed disease. U.S. Department of Agriculture Farmer's Bulletin No. 1054, Issued July 1919, Revised November 1936
54. Gardiner MR, Linto AC, Aplin THE (1969) Toxicity of Swainsona canescens for sheep in western Australia. Aust J Agric Res 20:87–97
55. Huang YQ, Zhang EY, Pan WF (2003) Current status of locoweed toxicity. Shandong Sci 16:34–39
56. Jones TC, Hunt RD, King NW (1997) Veterinary pathology. Williams and Wilkins, Baltimore, pp 1–1392
57. James LF, Van-Kampen KR, Hartley WJ (1970) Comparative pathology of Astragalus (locoweed) and Swainsona poisoning in sheep. Path Vet 7:116–125
58. Panter KE, James LF, Stegelmeier BL, Ralphs MH, Pfister JA (1999) Locoweeds: effects on reproduction in livestock. J Nat Toxins 8:53–62
59. Colegate SM, Dorling PR, Huxtable CR (1979) A spectroscopic investigation of swainsonine: an alpha-mannosidase inhibitor isolated from Swainsona canescens. Aust J Chem 32:2257–2264
60. Molyneux RJ, James LF (1982) Loco intoxication: indolizidine alkaloids of spotted locoweed (Astragalus lentiginosus). Science 216:190–191
61. Hartley WJ (1971) Some observations on the pathology of Swainsona spp. poisoning in farm livestock in Eastern Australia. Acta Neuropathol 18:342–355
62. Dorling PR, Huxtable CR, Vogel P (1978) Lysosomal storage in Swainsona spp. toxicosis: an induced mannosidosis. Neuropath Appl Neuro 4:285–295
63. Wang Q, Nagao H, Li Y, Wang H-S, Kakishima M (2006) Embellisia oxytropis, a new species isolated from Oxytropis kansuensis in China. Mycotaxon 95:255–260
64. Braun K, Romero J, Liddell C, Creamer R (2003) Production of swainsonine by fungal endophytes of locoweed. Mycol Res 107:980–988
65. Pryor BM, Creamer R, Shoemaker RA, McLain-Romero J, Hambleton S (2009) Undifilum, a new genus for endophytic Embellisia oxytropis and parasitic Helminthosporium bornmuelleri on legumes. Botany 87:178–194

66. Yu Y, Zhao Q, Wang J, Wang J, Wang Y, Song Y, Geng G, Li Q (2010) Swainsonine-producing fungal endophyte from major locoweed species in China. Toxicon 56:330–338
67. Baucom D, Romero M, Belfon R, Creamer R (2012) Two new species of Undifilum, the swainsonine producing fungal endophyte, from Astragalus species of locoweed in the United States. Botany 90:866–875
68. Oldrup E, McLain-Romero J, Padilla A, Moya A, Gardner D, Creamer R (2010) Localization of endophytic Undifilum fungi in locoweed seed and influence of environmental parameters on a locoweed in vitro culture system. Botany 88:512–521
69. Ralphs MH, Creamer R, Baucom D, Gardner DR, Welsh SL, Graham JD, Hart C, Cook D, Stegelmeier BL (2008) Relationship between the endophyte Embellisia spp. and the toxic alkaloid swainsonine in major locoweed species (Astragalus and Oxytropis). J Chem Ecol 34:32–38
70. Gardner DR, Molyneux RJ, Ralphs MH (2001) Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (Oxytropis spp.). J Agric Food Chem 49:4573–4580
71. Cook D, Gardner DR, Ralphs MH, Pfister JA, Welch KD, Green BT (2009) Swainsonine concentrations and endophyte amounts of Undifilum oxytropis in different plant parts of Oxytropis sericea. J Chem Ecol 35:1272–1278
72. Cook D, Gardner DR, Grum D, Pfister JA, Ralphs MH, Welch KD, Green BT (2011) Swainsonine and endophyte relationships in Astragalus mollissimus and Astragalus lentiginosus. J Agric Food Chem 59:1281–1287
73. Cook D, Shi L, Gardner DR, Grum D, Welch KD, Ralphs MH (2012) Influence of phenological stage on swainsonine and endophyte concentrations in Oxytropis sericea. J Chem Ecol 38:195–203
74. Achata BJ, Creamer R, Gardner D (2012) Seasonal changes in Undifilum colonization and swainsonine content of locoweeds. J Chem Ecol 38:486–495
75. Afkhami ME, Rudgers JA (2008) Symbiosis lost: imperfect vertical transmission of fungal endophytes in grasses. Am Nat 172:405–416
76. Ralphs MH, Cook D, Gardner DR, Grum DS (2011) Transmission of the locoweed endophyte to the next generation of plants. Fungal Ecol 4:251–255
77. Roylance JT, Hill NS, Agee CS (1994) Ergovaline and peramine production in endophyte-infected tall fescue: independent regulation and effects of plant and endophyte genotype. J Chem Ecol 20:2171–2183
78. Easton HS, Latch GCM, Tapper BA, Ball OJ (2002) Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. Crop Sci 42:51–57
79. Grum DS, Cook D, Gardner DR, Roper JM, Pfister JA, Ralphs MH (2012) Influence of seed endophyte amounts on swainsonine concentrations in Astragalus and Oxytropis locoweeds. J Agric Food Chem 60:8083–8089
80. Cook D, Grum D, Gardner DR, Welch KD, Pfister JA (2013) Influence of endophyte genotype on swainsonine concentrations in Oxytropis sericea. Toxicon 61:105–111
81. Pfister JA, Stegelmeier BL, Gardner DR, James LF (2003) Grazing of spotted locoweed (Astragalus lentiginosus) by cattle and horses in Arizona. J Anim Sci 81:285–293
82. Ralphs MH, Gardner DR, Graham JD, Greathouse G, Knight AP (2002) Clipping and precipitation influences on locoweed vigor, mortality, and toxicity. J Range Manage 55:394–399
83. Parker JE (2008) Effects of insect herbivory by the four-lined locoweed weevil, Cleonidus trivittaus (Say) (Coleoptera: Curculionidae), on the alkaloid swainsonine in locoweed Astragalus mollissimus and Oxytropis sericea. MS thesis, New Mexico State University, New Mexico, p 164
84. Valdez Barillas JR, Paschke MW, Ralphs MH, Child RD (2007) White locoweed toxicity is facilitated by a fungal endophyte and nitrogen-fixing bacteria. Ecol 88:1850–1856
85. Delaney KJ, Klypina N, Maruthavanan J, Lange D, Sterling TM (2011) Locoweed dose responses to nitrogen: positive for biomass and primary physiology, but inconsistent for an alkaloid. Am J Bot 98:1956–1965

86. Vallotton AD, Murray LW, Delaney KJ, Sterling TM (2012) Water deficit induces swainsonine of some locoweed taxa, but with no swainsonine–growth trade-off. Acta Oecol 43:140–149
87. McLain-Romero J, Creamer R, Zepeda H, Strickland J, Bell G (2004) The toxicosis of Embellisia fungi from locoweed (Oxytropis lambertii) is similar to locoweed toxicosis in rats. J Anim Sci 82:2169–2174
88. Dorling PR, Colegate SM, Huxtable CR (1989) Toxic species of the plant genus Swainsona. In: James LF, Elbein AD, Molyneux RJ, Warren CD (eds) Swainsonine and Related Glycosidase Inhibitors. Iowa State University Press, Ames, pp 14–22
89. de Balogh KK, Dimande AP, van derLJJ, Molyneux RJ, Naudé TW, Welman WG (1999) A lysosomal storage disease induced by Ipomoea carnea in goats in Mozambique. J Vet Diagn Invest 11:266–273
90. Barbosa RC, Riet-Correa F, Medeiros RMT, Lima EF, Barros SS, Gimeno EJ, Molyneux RJ, Gardner DR (2006) Intoxication by Ipomoea sericophylla and Ipomoea riedelii in goats in the state of Paraíba, Northeastern Brazil. Toxicon 47:371–379
91. Dantas AFM, Riet-Correa F, Gardner DR, Medeiros RM, Barros SS, Anjos BL, Lucena RB (2007) Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of Turbina cordata in Northeastern Brazil. Toxicon 49:111–116
92. Colodel EM, Gardner DR, Zlotowski P, Driemeier D (2002) Identification of swainsonine as a glycoside inhibitor responsible for Sida carpinifolia poisoning. Vet Hum Toxicol 44:177–178
93. Patrick M, Adlard MW, Keshavarz T (1993) Production of an indolizidine alkaloid, swainsonine by the filamentous fungus, Metarhizium anisopliae. Biotechnol Lett 15:997–1000
94. Schneider M, Ungemach F, Broquist H, Harris TM (1983) (1S,2R,8R,8aR)-1,2,8 trihydroxyoctahydroindolizidne (swainsonine), an α-mannosidase inhibitor from Rhizoctonia leguminicola. Tetrahedron 39:29–32
95. Cook D, Beaulieu WT, Mott IW, Riet-Correa F, Gardner DR, Grum D, Pfister JA, Clay K, Marcolongo-Pereira C (2013) Production of the alkaloid swainsonine by a fungal endosymbiont of the Ascomycete order Chaetothyriales in the host Ipomoea carnea. J Agric Food Chem 61:3797–3803

Chapter 3
Lectins from Medicinal Plants: Bioeffectors with Diverse Activities

Alexander V. Timoshenko, Irina V. Gorudko and Hans-Joachim Gabius

Abstract Lectins represent a family of glycan-binding proteins that are differentially expressed in various plant tissues and organs. As a component of traditional herbs, some purified plant lectins are known to possess immunomodulatory, cytotoxic, and anticancer activities with a potential biomedical application. In this chapter, we summarize our procedures for lectin isolation from medicinal plants and methods for lectin screening and biotesting based on a variety of cellular responses (cell aggregation, generation of hydrogen peroxide, and secretion of vascular endothelial growth factor C). The importance of detecting and characterizing lectins in herbal preparations is discussed in the context of safety and efficacy of lectin-based phytotherapeutical approaches.

Keywords Cancer · Innate immunity · Lectins · Medicinal plants · Phytochemicals

3.1 Introduction

Cellular glycans are increasingly recognized as a versatile basis to store and transmit biological information [1]. Due to this appreciation of sugars as the third alphabet of life forming the sugar code [1, 2], the ways sugar-encoded information is translated into cellular effects have become an attractive study area. A key role in this process is played by lectins, glycan-binding proteins, which are neither glycoenzymes such as glycosyltransferases or glycosidases, nor glycan-specific antibodies, nor transporters for free glycans [2]. Starting with H. Stillmark's pioneering work on a respective activity in *Ricinus communis* in 1888, plants have proven to be a rich

A. V. Timoshenko (✉)
Department of Biology, Western University, London,Ontario N6G 5B7, Canada
e-mail: atimoshe@uwo.ca

I. V. Gorudko
Department of Biophysics, Belarusian State University,
220030 Minsk, Belarus

H.-J. Gabius
Institute of Physiological Chemistry, Faculty of Veterinary Medicine,
Ludwig-Maximilians-University, 80539 Munich, Germany

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_3

Table 3.1 The list of medicinal plants specified in 2012 US Pharmacopeia that contain lectins based on a search of peer-reviewed sources on PubMed

Latin name of plants	PubMed hits[a] (all/lectin)	Lectin MW, kDa	Nominal glycan-binding specificity	References
Aesculus hippocastanum	368/2	132	O-glycan	[12]
Allium sativum	4,119/75	25, 24, 48	α(1→3)Man	[13–15]
Curcuma longa	1,664/11	19.6, 20.3, 404	Man	[16]
Glycine max	21,088/923	110	GalNAc	[17]
Trifolium pratense	1,192/5	ND	ND	[18][b]
Urtica dioica	332/49	8.3–9.5	(GlcNAc)n	[19, 20]
Vitis vinifera	1,469/2	11.9–13.2	p-nitrophenyl β-D-Gal/Glc	[21]
Withania somnifera	521/3	30	ND	[22]
Zingiber officinale	1,724/4	16.2	Man	[23]

[a] The search was performed using first Latin names of the plants as key words and then in their combination with "lectin" as second key word
[b] In silico analysis
ND not determined

source for lectins, then referred to as phytohaemagglutinins because of their capacity to agglutinate erythrocytes in a carbohydrate-dependent manner [3]. The highest concentration of lectins is usually detected in seeds, for example with 2.1 g/100 g in beans [3]. With purification yields in this range, it is evident why these glycan-binding proteins have become a popular tool for structural research [1–3].

The enormous toxicity of certain lectins, especially the AB toxins such as ricin, and also other activities on mammalian cells such as mitogenicity or the simple capacity to define aspects of the glycophenotype have made plant lectins study objects in biomedicine. Owing to their stability, they maintain their active form in vivo even after consuming a lectin-containing meal, e.g., with roasted peanuts [4]. Of interest in this context, purified plant lectins are known to affect aspects of functional activity of mammalian cells that are associated with immunomodulation (e.g., mitogenic stimulation of lymphocytes, activation of innate immunity, production of cytokines, and growth factors) [5–8], by virtue of binding to distinct glycans, e.g., α 2,3/6-sialylated N- and O-glycans. Detailed glycan mapping, with plant lectins as sensors, in combination with tissue lectins, has been instrumental to define, for instance, the master-regulator potency of a tumor suppressor which downregulates α 2,6-sialylation [9, 10]. Along this line, the significance and role of a lectin component in medicinal plants can deserve attention in phytopharmacology, despite the relatively low content of lectins in traditional pharmacopoeial materials, e.g., leaves and flowers. As a consequence, available information about lectins in medicinal plants that are listed in national pharmacopeias is rather limited [11]. In particular, a PubMed-based search (December 2012) on lectins (also using the synonymous term "agglutinin") in 43 herbs from Dietary Supplements Official Monographs of the 2012 US Pharmacopeia has revealed that information about lectins in medicinal plants is available only for ~ 21 % of the listed herbs (Table 3.1) [12–23].

Of interest in this context, the market in Central and Eastern Europe offers a variety of so-called lectin-containing herbal teas that are based on medicinal plants such

as *Agastache rugosa* (wrinkled giant hyssop), *Calendula officinalis* (pot marigold), *Hypericum perforatum* (St. John's wort), *Salvia officinalis* (sage), *Melissa officinalis* (lemon balm), *Mentha piperita* (peppermint), *Nepeta cataria* (catmint), and *Zea mays* (corn silk). To the best of our knowledge, no peer-reviewed publications on lectin composition in the aqueous extracts of the above-mentioned plants, except for *Z. mays* seeds but not silk, are available in PubMed, and thus the significance of leaf lectins is unclear. In fact, recent studies have identified several leaf lectins with potential antimicrobial and antiproliferative properties in medicinal plants including *Morus* and *Schinus* species [24, 25]. To date, only one leaf lectin, a galactoside-binding lectin (agglutinin) from mistletoe leaves (*Viscus album* agglutinin, VAA), was examined rather rigorously as a drug. Based on a purely spiritual concept, mistletoe extracts had been suggested to be able to cure cancer by acting on the dysbalance between nonmaterial spheres within the patient's personality [26]. Biotesting of the purified galactose-binding lectin from clinically approved mistletoe extracts had shown that its application in ng quantities had diverse immunomodulatory effects both in vitro and in vivo, including the increase in secretion of cytokines such as interleukin-6, which has a potential to stimulate tumor growth [7, 27, 28]. When increasing the concentration, mistletoe lectins were shown to be cytotoxic for tumor and normal cells alike [29]. Overall, these studies indicate that many medicinal plants may contain biologically active lectins that warrant investigation. To this end, methods for lectin isolation and characterization must be established and optimized. The purpose of this chapter is to summarize our ongoing work in this field, first illustrating the procedure for lectin isolation (Sect. 3.2), then the assays used for biotesting of plant lectins in different cellular systems, e.g., isolated human blood cells (Sects. 3.3 and 3.4) and cancer cells (Sects. 3.5 and 3.6). Special aspects covered are applicability of lectins from medicinal plants and the specification of different levels of lectin-mediated modulation of selected cellular responses, such as cell aggregation (Sect. 3.3), hydrogen peroxide generation (Sect. 3.4), and vascular endothelial growth factor C (VEGF-C) secretion (Sect. 3.5). Based on this, it will be discussed whether treatment with a lectin immunomodulator can cause harmful effects in vivo (Sect. 3.6).

3.2 Isolation of Lectins from Medicinal Plants

A gold standard for lectin isolation is glycan-based affinity chromatography, which will need to be optimized for each protein studied. Such work is outlined in this section for the mistletoe lectin VAA, with an eye on yield [30]. To start with an important aspect of this technique, the proper matrix should be selected and substituted with covalently attached glycans using a spacer of sufficient length and flexibility to bind lectins. To isolate the galactoside-binding lectin from mistletoe leaves, we have employed Sepharose 4B with immobilized lactose. The preparation of this affinity matrix included activation (50 mL, rinsed with 1 L of distilled H_2O and then 0.5 M Na_2CO_3 buffer, pH 11) with 6 mL divinyl sulfone for 90 min. The divinyl sulfone-activated resin was then mixed with a solution of 20% lactose and left overnight

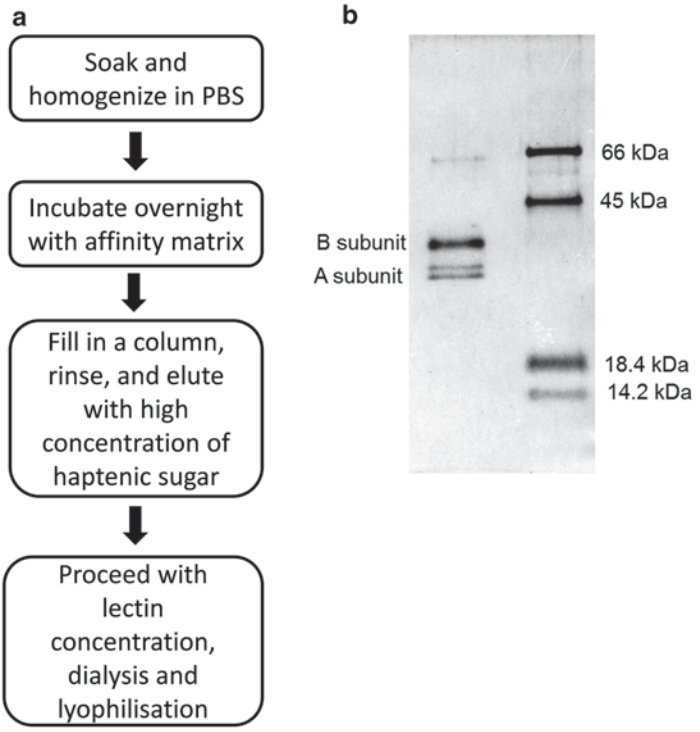


Fig. 3.1 Isolation and characteristics of Gal-binding lectin from mistletoe leaves. **a** Main steps of the lectin purification. **b** SDS-PAGE gel of purified VAA showing the presence of glycan-binding B subunit (34 kDa) and two closely related toxic subunits A1 (28.5 kDa) and A2 (29.5 kDa); protein standards were bovine serum albumin (66 kDa), egg albumin (45 kDa), β-lactoglobulin (18.4 kDa), and α-lactoglobulin (14.2 kDa)

under gentle rotation or stirring for lactose immobilization. Final steps of Sepharose 4B-lactose resin preparation required consecutive washes with distilled H_2O, 0.5 M $NaHCO_3$ buffer (pH 8.5), 2 h treatment with 4% β-mercaptoethanol to block any residual activated groups, and finally extensive washes with distilled H_2O and then 20 mM phosphate-buffered saline (PBS, pH 7.2) to completely remove any reagents.

Figure 3.1a shows the flow chart for isolation of VAA using the Sepharose 4B-lactose affinity matrix prepared as described above. All steps were performed at room temperature if not otherwise indicated. First, 100 g of dried leaves of *V. album* from a local drug store were macerated overnight in 400 mL of 20 mM PBS (pH 7.2), homogenized with additional 200 mL of PBS in a blender for 3 min, and filtered through a Buchner funnel. Second, the filtrate was centrifuged at 14,000 rpm for 20 min at 4 °C, in a Beckman J2-21 centrifuge (JA-14 rotor), and the supernatant was mixed with 30 mL of the Sepharose 4B-lactose matrix. The mixture was incubated overnight in a cold room (4 °C) with gentle shaking. Third, a glass column (2.5 × 7 cm) was filled with the gel suspension, washed with 600 mL PBS (flow rate of 150 mL/h), and, after washing thoroughly, VAA was eluted (rate of 50 mL/h) with 150 mL of

0.3 M lactose in PBS (pH 7.2). Fourth, the eluate was concentrated down to 10 mL in an Amicon ultrafiltration cell 8200 (YM5 membrane), dialyzed first against PBS (3 days with daily buffer exchange of 2 L) and then distilled H_2O (day 4), aliquoted at 500 μg, frozen and lyophilized. The entire isolation procedure lasted 1 week and can result in 4–6 mg/100 g d.w. of mistletoe leaves. The purity of the lectin attained was confirmed by SDS-PAGE as described by Laemmli [31] using vertical slab gels ($10 \times 7 \times 0.1$ cm) that combined a 4% stacking gel (length is 1.5 cm) and 10% separating gel (length is 8.5 cm). The gels were run at a constant current of 20 mA in a Mini-PROTEAN system and stained using $AgNO_3$ as described elsewhere [32]. This isolation method produced the heterodimer with molecular weight of 62 kDa that consists of a carbohydrate-binding B subunit (34 kDa) and a mixture of two slightly different toxic subunits A1 and A2 of 28.5 kDa and 29.5 kDa, respectively (Fig. 3.1b). The toxicity of A subunits is based on their ability to inactivate eukaryotic ribosomes by cleaving a single N-glycosidic bond at adenosine-4324 of 28S rRNA [33]. Further analysis of the lectin revealed a concentration-dependent equilibrium in the quaternary structure and preferential lectin activity to the so-called Tyr site in the dimer [34, 35].

3.3 Lectin-Induced Cell Aggregation Assay

Cell aggregation or agglutination activity is a 'natural' property of many lectins, because they can serve as molecular bridges between cell surface glycans of contacting cells, if harbouring more than one glycan-binding site as VAA does [36]. As such, the lectin-induced aggregation assay senses the accessibility of reactive glycans on the cell surface. To run this assay, lectins (usually 1–50 μg/mL) are added to the suspension of isolated human/animal cells ($1–5 \times 10^6$ cells/mL) in a balanced saline solution (PBS or HBSS). The formation of cell aggregates can be detected microscopically and spectrophotometrically by recording changes in the turbidity of cell suspensions [37]. Microscopic detection, although mostly qualitative, is however required to ascertain the formation of cell aggregates (Fig. 3.2a–c). Turbidimetric cell aggregation assay is quantitative and requires respective equipment, i.e., cell aggregometers, which are available from several suppliers such as SOLAR (Minsk, Belarus) or Chrono-Log (Havertown, PA), or any spectrophotometer with stirring and temperature-controlled cell.

Figure 3.2d, e (trace 1) show the courses of aggregation of human neutrophils (2×10^6 cells/mL) by two lectins with different glycan-binding properties, i.e., VAA (5 μg/mL) and wheat germ agglutinin (WGA) (50 μg/mL). Both lectins induced increase in light transmission of cell suspensions at 560 nm. This increase correlates well with microscopic observations of either individual cells or cell aggregates in the absence and presence of lectins, respectively (Fig. 3.2a, b). The glycan-binding specificity of lectins can also be measured in an aggregation assay by adding inhibitory (or haptenic) sugars prior or together with lectin(s). For example, as seen in Fig. 3.2d, e (trace 2), the aggregation response to VAA is inhibited by lactose

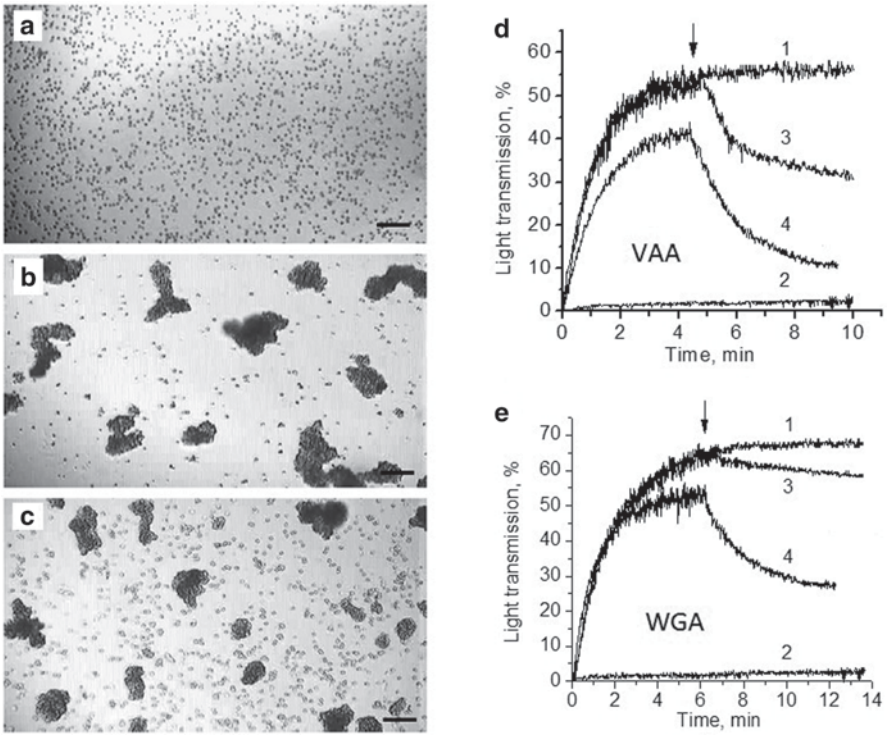


Fig. 3.2 Microscopical and turbidimetric detection of lectin-induced aggregation of human neutrophils. **a** Control suspension of neutrophils (2×10^6 cells/mL). **b** neutrophil aggregates formed in response to VAA (5 μg/mL) at 10–15 min corresponding to the plateau phase of the aggregation response. **c** VAA-induced neutrophil aggregates after addition of lactose to a concentration of 55 mM to cell aggregates at the plateau phase. **d** and **e** aggregation kinetics of human neutrophils recorded as changes in the light transmission of cell suspensions at 560 nm after the addition of VAA (5 μg/mL) and WGA (50 μg/mL), respectively: *1* a regular kinetics of lectin-induced cell aggregation, *2* inhibition of cell aggregation by haptenic sugars added before lectins (55 mM lactose to inhibit VAA and 100 mM GlcNAc to inhibit WGA), *3* formation of HSR contacts as proven by adding haptenic sugar to cell aggregates (indicated by *arrow*), *4* inhibition of HSR contacts in the presence of N-ethylmaleimide (50 μM) as revealed by cell aggregate dissociation induced by haptenic sugars (reproduced with permission from [37]). Bar = 100 μm (**a–c**)

(55 mM), whereas the aggregation response to GlcNAc-binding WGA is inhibited by GlcNAc (100 mM). Haptenic sugars can also be added in the course of the aggregation reaction, stopping cell aggregation or inducing dissociation of cell aggregates (Fig. 3.2d, e, trace 3). Remarkably, not all lectin-induced cell aggregates can be dissociated by haptenic sugars, indicating that additional intercellular contacts could be formed in response to lectins as demonstrated earlier [38–41]. Such lectin-inducible haptenic-sugar-resistant (HSR) contacts resulted from activation of transmembrane signaling in cells, because they can be inhibited by compounds [39, 40] that affect signaling pathways involved in cell adhesion [42]. For example, a very efficient inhibitor of HSR contacts is the sulfhydryl-blocking reagent N-ethylmaleimide, addition of which leads to dissociation of cell aggregates in the presence of

haptenic sugars (Fig. 3.2d, e, trace 4). It should be noted that lectin-induced increase in cell adhesion and expression of adhesion receptors was documented for human eosinophils and monocytes [43, 44]. Thus, analysis of cell aggregation and dissociation responses enables detecting both the presence of lectin-binding glycans on the cell surface and the ability of lectins to link counterreceptors, a process which can lead to stimulation of transmembrane signaling in cells. This approach is especially important for lectins from medicinal plants, because it provides a powerful and rapid method for screening lectins with potential immunomodulatory activity, for testing immune cells such as neutrophils and lymphocytes. To quantify the extent of cell aggregation and disaggregation, three parameters are suited, i.e., the maximal rate of cell aggregation based on the slope of the aggregation curve, the maximal extent of aggregation based on the light transmission plateau, and the stability of lectin-induced cell aggregates based on the response to haptenic sugars [37]. Interestingly, while there was a significant correlation between the aggregation rate and extent, the stability of lectin-induced aggregates, for obvious reasons, did not correlate in the majority of cases with those of the two conventional aggregation indices.

3.4 Lectin-Induced Generation of Hydrogen Peroxide by Human Neutrophils

Many plant lectins are able to activate plasma membrane NADPH oxidase, a pivotal enzyme complex of phagocytic cells (neutrophils, eosinophils, and macrophages) which generates superoxide anion radicals [45]. Superoxide radicals are dismutated spontaneously or enzymatically into H_2O_2, which is often used as an indicator of the phagocyte NADPH oxidase activity [6, 46]. The biological importance of this special oxidase is to make an effector in innate immunity available, because this reactive oxygen species contributes to the inactivation of pathogens taken up by phagocytosis [47]. The functionally active NADPH oxidase complex is assembled in cholesterol-enriched lipid rafts with plasma membrane flavocytochrome b558 (heterodimer of p22phox and gp91phox) and four cytoplasmic subunits (p40phox, p47phox, p67phox, and Rac) [48]. The only glycosylated component of this complex is gp91phox (NOX2), which is a key catalytic subunit and a founding member of NOX family of NADPH oxidases [48, 49]. We demonstrated that the sustainability of lectin-induced generation of H_2O_2 by human neutrophils depends on whether or not lectins interact with cytochrome b558, i.e., its glycosylated subunit gp91phox, and this interaction minimizes a requirement of presentation in lipid rafts [50]. This aspect of lectin activity can be disclosed by examining H_2O_2 generation by human neutrophils treated with drugs impairing the integrity of lipid rafts, e.g., with methyl-β-cyclodextrin which removes cholesterol from the cells without abolishing their viability [50].

A convenient assay of the phagocyte NADPH oxidase activity utilizes scopoletin, a fluorescent substrate of horseradish peroxidase (HRP) [6, 46]. To run this assay, neutrophils are used at a concentration of 1×10^6 cells/mL in PBS or HBSS (pH 7.2–7.3) containing 1 μM of scopoletin, 20 μg/ml of HRP, and 1 mM of NaN_3. Sodium azide is an optional component and included to inhibit endogenous myeloperoxidase and

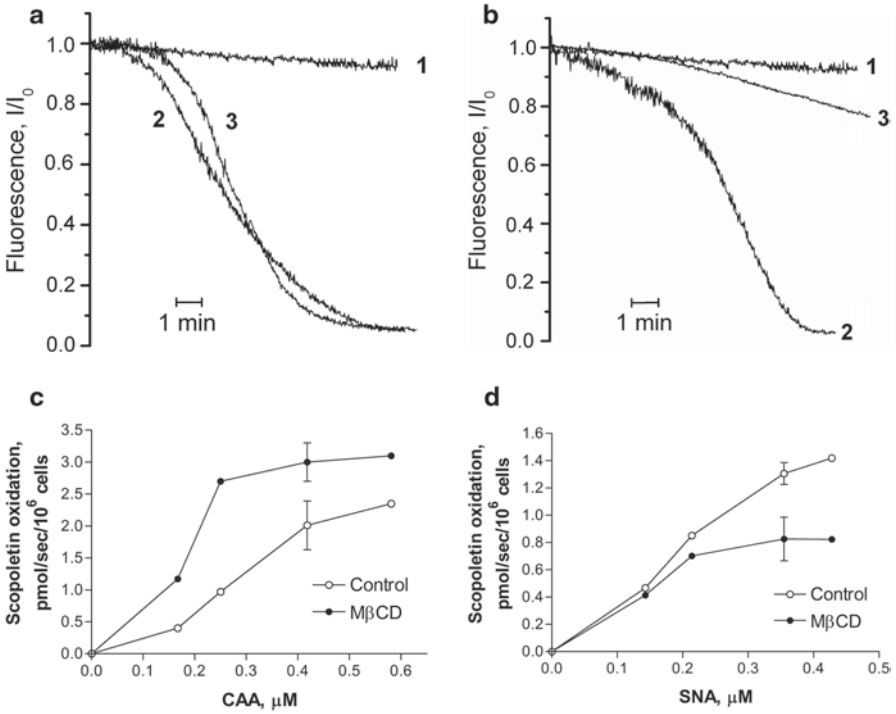


Fig. 3.3 Lectin-induced oxidation of scopoletin by human neutrophils as a measure of H_2O_2 generation. **a** and **b** Cell responses to GalNAc-binding lectin from *Caragana arborescens* (50 μg/mL) and NeuNAcα2,6Gal/GalNAc-binding lectin from *Sambucus nigra* (50 μg/mL): *1* basal oxidation, *2* lectin-induced oxidation, *3* lectin-induced oxidation in the presence of 10 mM methyl-β-cyclodextrin. **c** and **d** dose-dependent effects of lectins from *Caragana arborescens* (CAA) and *Sambucus nigra* (SNA) on scopoletin oxidation in the absence and presence of 10 mM methyl-β-cyclodextrin. Error bars were included for only one tested concentration on cells isolated from at least 3–5 donors. The reaction was performed in suspensions of human neutrophils in PBS, pH 7.2 (10^6 cells/mL) supplemented with 1 μM scopoletin, 20 μg/mL horseradish peroxidase, and 1 mM NaN_3 at 37 °C. (Reproduced with permission from [50])

catalase activity that provides better conditions for the detection of total H_2O_2 production. To start the reaction, a lectin is added (final concentration ranges from 1 to 50 μg/mL) to 1.5–2 mL of the stirred cell suspension at 37 °C, and the kinetics of scopoletin oxidation is recorded as a decrease in fluorescence intensity at 460 nm (excitation at 350 nm) (Fig. 3.3). The quantification of lectin-induced H_2O_2 generation yields two parameters; these are the maximal rate of scopoletin oxidation based on the slope of the kinetics and the lag-period, i.e., the initial time required to assemble NADPH oxidase complex. To infer whether lectins interact with gp91phox, the neutrophils are treated with methyl-β-cyclodextrin (extracting cholesterol from lipid microdomains; 10 mM) for 10 min at 37 °C and the regular response to lectins is measured. Methyl-β-cyclodextrin-induced inhibition of the lectin response suggests no direct interaction between lectin and flavocytochrome b558, while the sustained or increased H_2O_2 generation implies an interaction. Figure 3.3 shows the

corresponding kinetics and dose-dependent curves for two experiments with lectins from *Caragana arborescens* and *Sambucus nigra,* with opposite properties.

3.5 Lectin-Induced Secretion of VEGF-C by Cancer Cells

Binding of lectin with cells can induce the secretion of diverse types of immune modulators and cytokines having been noted above [27, 28]. This cellular response can be harmful in vivo as implied by an ongoing paradigmatic shift considering the tumor-promoting activity spectrum of some cytokines [51, 52]. To substantiate this concept, we present respective experimental data, first introducing a rather novel activity in this context. A dysregulated presence of growth factors required for angiogenesis and lymphangiogenesis is considered as a signature of cancer cells that require an efficient vascular system in tumors [53]. We showed that overproduction of VEGF-C, which is a major lymphangiogenic factor, is a feature of highly metastatic human breast cancer cell line MDA-MB-231 [54]. The level of VEGF-C in the cell culture medium may reach up to 10 ng/mL as can be readily measured by commercially available ELISA kits. Plant lectins with different glycan-binding properties were able to stimulate VEGF-C secretion by MDA-MB-231 cells, to a similar extent as human lectins (Fig. 3.4), what may also suggest a protumoral aspect of activity of these lectins. However, interpretation should be done cautiously for at least two reasons: First, there was evidence that other breast cancer cell lines (MCF7 and T-47D) showed differential responses to galectins and concanavalin A [55] and second, VEGF-C can mediate alternative functions in vivo affecting the permeability of blood vessels [56] and activating $\alpha^9\beta^1$ integrin and neuropilins that are involved in cell migration [57]. Thus, VEGF-C testing with cancer cells is an example of an assay for lectins from medicinal plants to gain insights on their biomedical potential. To decide on in vivo benefit or problems, animal models will provide salient information.

3.6 Tumor-Promoting Effects of Mistletoe Lectin In Vivo

In order to address the issue on potentially harmful effects of immunomodulation, a lectin can be tested at doses effective to increase cytokine levels. Such work has been done for the mistletoe lectin in two animal models. First, long-term VAA therapy was used to treat urinary bladder carcinomas in rats induced by N-methyl-N-nitrosourea or N-butyl-N-(4-hydroxybutyl)-nitrosamine [58, 59]. The standard regime of subcutaneous injections of VAA (1 ng/kg body weight, biweekly) was applied for 15 months. In both cases, no protection against chemically induced bladder carcinomas was noticed. In contrast, evidence for tumor stimulation was obtained. Second, short-term VAA therapy was applied to a breast cancer model in C3H/HeJ mice, which were transplanted with a metastatic C3L5 mammary adenocarcinoma cell line [52]. This model provides a convenient in vivo tool to monitor

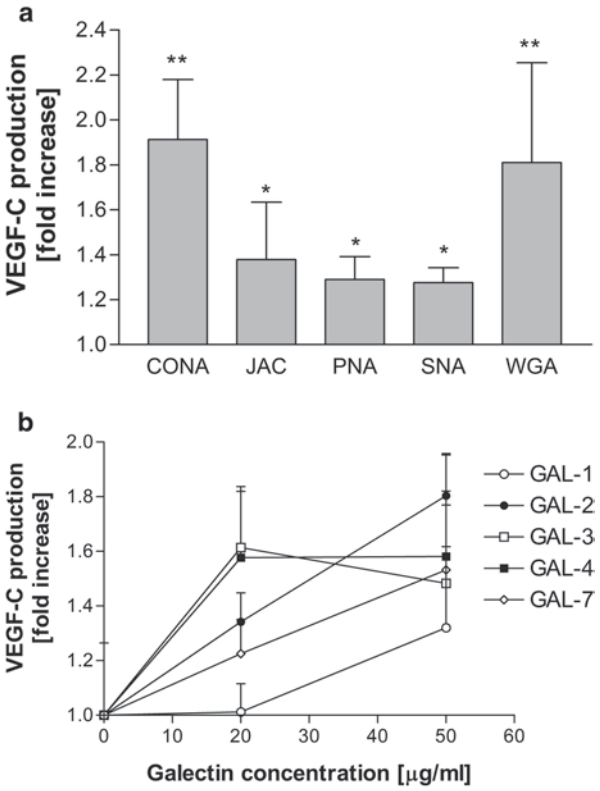


Fig. 3.4 Effect of plant lectins and human galectins on VEGF-C production by MDA-MB-231 cells. **a** Accumulation of VEGF-C in the cell culture medium (DMEM) of 80–90 % confluent culture in response to plant lectins (5 μg/ml) at 24 h (ConA concanavalin A, *JAC* jacalin, PNA peanut agglutinin, SNA *Sambucus nigra* agglutinin, WGA wheat germ agglutinin). **b** concentration-dependent secretion of VEGF-C by the cells in response to human galectins (GAL) at 24 h. The basal production of VEGF-C by untreated cells was 1127 ± 343 pg/ml ($n = 4$). *$p < 0.05$, **$p < 0.001$. (Reproduced with permission from [55])

tumor growth over a period of few weeks. While tumor growth was inhibited by the routine intraperitoneal administration of interleukin-2, subcutaneous injections of VAA (1 ng/kg body weight biweekly) promoted tumor growth and the incidence of lung metastasis (Fig. 3.5). The C3L5 cell line, which was used in this study, is characterized by a high metastatic activity resulting most likely from high cyclooxygenase-2, eNOS, and VEGF-C/VEGF-D expression [52, 60] that may make it sensitive to lectin-induced cytokines. Potential of growth stimulation in human histocultures [29] further adds to the notion that the actual physiological context determines the outcome of lectin application. It is therefore mandatory to rigorously exclude any risk potential of an immunomodulatory application (*primum non nocere*).

3.7 Conclusions

Medicinal plants in many cases contain lectins. They can readily be isolated using affinity chromatography, as demonstrated for a galactoside-binding lectin from mistletoe leaves. As a part of aqueous extracts, these lectins may contribute to the

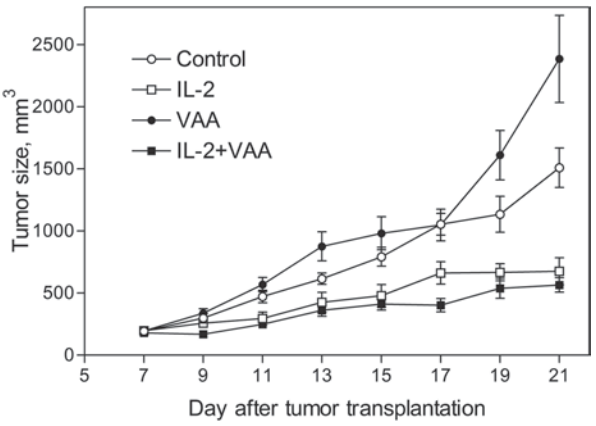


Fig. 3.5 Effects of intraperitoneal injection of interleukin-2 (IL-2, 6×10^4 IU every 8 h during 5 days, followed by a 4-day recess and then a similar 5-day round) and subcutaneous (s.c.) injections of VAA (1 ng/kg body weight, biweekly), as well as their combination on tumor growth after s.c. inoculation of 5×10^5 C3L5 cells in C3H/HeJ female mice. Starting at 1 week after tumor transplantation, the minimal and maximal diameters (*a* and *b*) of the tumors were measured on alternate days using digital calipers and the tumor volume was calculated according to the equation $V = 0.52a^2b$. Data indicate mean ± standard error ($n = 6$–10). (Reproduced with permission from [52])

activity profile of herbal medications, which needs to be thoroughly assessed. As instructive examples, different features of activity can be examined, e.g., using the aggregation assay in conjunction with inhibitory sugars and assays measuring the production of regulatory molecules such as reactive oxygen species and growth factors. In vivo work, as shown, is essential to identify and exclude harmful consequences that may arise from an administration of lectin-containing extracts. The set of methods that we have described in this chapter can thus be applied for screening and characterizing lectins in medicinal plants, with the aim of lectin-based assessment and standardization of herbal extracts, required to ensure optimal efficacy and herbal medicine safety.

References

1. Gabius HJ (ed) (2009) The sugar code. Fundamentals of glycosciences. Wiley-VCH, Germany
2. Gabius HJ, André S, Jiménez-Barbero J, Romero A, Solís D (2011) From lectin structure to functional glycomics: principles of the sugar code. Trends Biochem Sci 36:298–313
3. Rüdiger H, Gabius HJ (2001) Plant lectins: occurrence, biochemistry, functions and applications. Glycoconj J 18:589–613
4. Wang Q, Yu LG, Campbell BJ, Milton JD, Rhodes JM (1998) Identification of intact peanut lectin in peripheral venous blood. Lancet 352:1831–1832

5. Kilpatrick DC (1998) Mechanisms and assessment of mitogenesis: an overview. Methods Mol Med 9:365–378
6. Timoshenko AV, Kayser K, Gabius HJ (1998) Lectin-triggered superoxide/H_2O_2 and granule enzyme release from cells. Methods Mol Med 9:441–451
7. Gabius HJ (2001) Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie 83:659–666
8. Shanmugham LN, Castellani ML, Salini V, Falasca K, Vecchiet J, Conti P, Petrarca C (2006) Relevance of plant lectins in human cell biology and immunology. Riv Biol 99:227–249
9. André S, Sanchez-Ruderisch H, Nakagawa H, Buchholz M, Kopitz J, Forberich P, Kemmner W, Böck C, Deguchi K, Detjen KM, Wiedenmann B, von Knebel DM, Gress TM, Nishimura S, Rosewicz S, Gabius HJ (2007) Tumor suppressor $p16^{INK4a}$: modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J 274:3233–3256
10. Amano M, Eriksson H, Manning JC, Detjen KM, André S, Nishimura S, Lehtiö J, Gabius HJ (2012) Tumour suppressor $p16^{INK4a}$: anoikis-favouring decrease in N/O-glycan/cell surface sialylation by down-regulation of enzymes in sialic acid biosynthesis in tandem in a pancreatic carcinoma model. FEBS J 279:4062–4080
11. Timoshenko AV (2003) Lectins in medicinal plants. Proc Natl Acad Sci Belarus Biol N2:104–113
12. Antoniuk VO (1992) Isolation of lectin from horse chestnut (*Aesculus hippocastanum* L.) seeds and study of its interaction with carbohydrates and glycoproteins. Ukr Biokhim Zh 64:47–52
13. Kaku H, Goldstein IJ, van Damme EJ, Peumans WJ (1992) New mannose-specific lectins from garlic (*Allium sativum*) and ramsons (*Allium ursinum*) bulbs. Carbohydr Res 229:347–353
14. van Damme EJ, Smeets K, Torrekens S, van Leuven F, Goldstein IJ, Peumans WJ (1992) The closely related homomeric and heterodimeric mannose-binding lectins from garlic are encoded by one-domain and two-domain lectin genes, respectively. Eur J Biochem 206:413–420
15. Dam TK, Bachhawat K, Rani PG, Surolia A (1998) Garlic (*Allium sativum*) lectins bind to high mannose oligosaccharide chains. J Biol Chem 273:5528–5535
16. Boonmee A, Srisomsap C, Chokchaichamnankit D, Karnchanatat A, Sangvanich P (2011) A proteomic analysis of *Curcuma comosa* Roxb. rhizomes. Proteome Sci 9:43. doi:10.1186/1477–5956-9–43
17. Lis H, Sharon N (1972) Soybean (*Glycine max*) agglutinin. Methods Enzymol 28:360–365
18. Gubaidullin II, Baimiev AK, Baimiev AK, Chemeris AV (2007) The carbohydrate-binding sequences in lectins of the clovers *Trifolium repens, T. pratense,* and *T. trichocephalum*. Russ J Genet 43:376–380
19. Shibuya N, Goldstein IJ, Shafer JA, Peumans WJ, Broekaert WF (1986) Carbohydrate binding properties of the stinging nettle (*Urtica dioica*) rhizome lectin. Arch Biochem Biophys 249:215–224
20. Does MP, Ng DK, Dekker HL, Peumans WJ, Houterman PM, Van Damme EJ, Cornelissen BJ (1999) Characterization of *Urtica dioica* agglutinin isolectins and the encoding gene family. Plant Mol Biol 39:335–347
21. Berthier L, Marchal R, Debray H, Bonnet E, Jeandet P, Maujean A (1999) Isolation of isolectins from *Vitis vinifera* L. Cv. Chardonnay grape berries. J Agric Food Chem 47:2193–2197
22. Ghosh M (2009) Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. Fitoterapia 80:91–95
23. Chen Z, Kai G, Liu X, Lin J, Sun X, Tang K (2005) cDNA cloning and characterization of a mannose-binding lectin from *Zingiber officinale* Roscoe (ginger) rhizomes. J Biosci 30:213–220
24. Deepa M, Priya S (2012) Purification and characterization of a novel anti-proliferative lectin from *Morus alba* L. leaves. Protein Pept Lett 19:839–845
25. Gomes FS, Procópio TF, Napoleão TH, Coelho LC, Paiva PM (2013) Antimicrobial lectin from *Schinus terebinthifolius* leaf. J Appl Microbiol 114:672–679
26. Burkhard B (2000) Anthroposophische Arzneimittel. Eine kritische Betrachtung. GOVI-Verlag, Eschborn

27. Hajto T, Hostanska K, Gabius HJ (1989) Modulatory potency of the β-galactoside-specific lectin from mistletoe extract (Iscador®) on the host defense system in vivo in rabbits and patients. Cancer Res 49:4803–4808
28. Hajto T, Hostanska K, Frei K, Rordorf C, Gabius HJ (1990) Increased secretion of tumor necrosis factor-α, interleukin-1, and interleukin-6 by human mononuclear cells exposed to the β-galactoside-specific lectin from clinically applied mistletoe extract. Cancer Res 50:3322–3326
29. Gabius HJ, Darro F, Remmelink M, André S, Kopitz J, Danguy A, Gabius S, Salmon I, Kiss R (2001) Evidence for stimulation of tumor proliferation in cell lines and histotypic cultures by clinically relevant low doses of the galactoside-binding mistletoe lectin, a component of proprietary extracts. Cancer Invest 19:114–126
30. Gabius HJ (1990) Influence of type of linkage and spacer on the interaction of β-galactoside-binding proteins with immobilized affinity ligands. Anal Biochem 189:91–94
31. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
32. Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99
33. Endo Y, Tsurugi K, Franz H (1988) The site of action of the A-chain of mistletoe lectin I on eukaryotic ribosomes: the RNA N-glycosidase activity of the protein. FEBS Lett 231:378–380
34. Jiménez M, Sáiz JL, André S, Gabius HJ, Solís D (2005) Monomer/dimer equilibrium of the AB-type lectin from mistletoe enables combination of toxin/agglutinin activities in one protein: analysis of native and citraconylated proteins by ultracentrifugation/gel filtration and cell biological consequences of dimer destabilization. Glycobiology 15:1386–1395
35. Jiménez M, André S, Siebert HC, Gabius HJ, Solís D (2006) AB-type lectin (toxin/agglutinin) from mistletoe: differences in affinity of the two galactoside-binding Trp/Tyr-sites and regulation of their functionality by monomer/dimer equilibrium. Glycobiology 16:926–937
36. Mikeska R, Wacker R, Arni R, Singh TP, Mikhailov A, Gabdoulkhakov A, Voelter W, Betzel C (2005) Mistletoe lectin I in complex with galactose and lactose reveals distinct sugar-binding properties. Acta Crystallogr F61:17–25
37. Gorudko IV, Buko IV, Cherenkevich SN, Polonetsky LZ, Timoshenko AV (2008) Lectin-induced aggregates of blood cells from patients with acute coronary syndromes. Arch Med Res 39:674–681
38. Timoshenko AV, Gorudko IV, Kaltner H, Cherenkevich SN, Gabius HJ (1997) Metabolic inhibitors as tools to delineate participation of distinct intracellular pathways in enhancement of lactose-induced dissociation of neutrophil and thymocyte aggregates formed by a plant lectin. Biochem Mol Biol Int 43:477–487
39. Timoshenko AV, Gorudko IV, Cherenkevich SN, Gabius HJ (1999) Differential potency of two crosslinking plant lectins to induce formation of haptenic-sugar-resistant aggregates of rat thymocytes by post-binding signaling. FEBS Lett 449:75–78
40. Timoshenko AV, Gorudko IV, Kaltner H, Gabius HJ (1999) Dissection of the impact of various intracellular signaling pathways on stable cell aggregate formation of rat thymocytes after initial lectin-dependent cell association of using a plant lectin as model and target-selective inhibitors. Mol Cell Biochem 197:137–145
41. Timoshenko AV, Gorudko IV, André S, Gabius HJ (2000) Cell-type dependence of stability modulation of lectin-initiated contacts by impairment of multivalent carbohydrate binding and intracellular signaling. Biosci Rep 20:199–209
42. Ruoslahti E, Obrink B (1996) Common principles in cell adhesion. Exp Cell Res 227:1–11
43. Fattah D, Page KR, Bezbaruah S et al (1996) A rapid activation assay for human eosinophils based on adhesion to immobilized ICAM-1, VCAM-1 and IgG. Cytokine 8:248–259
44. Tahara K, Nishiya K, Hisakawa N, Wang H, Hashimoto K (2003) Suppressive effect of iron on concanavalin A-induced multinucleated giant cell formation by human monocytes. Immunol Invest 32:229–243

45. Timoshenko AV, Gabius HJ (1993) Efficient induction of superoxide release from human neutrophils by the galactoside-specific lectin from *Viscum album*. Biol Chem Hoppe Seyler 374:237–243
46. Jones OTG, Hancock JT (1994) Assays of plasma membrane NADPH oxidase. Methods Enzymol 233:222–229
47. Segal BH, Grimm MJ, Khan AN, Han W, Blackwell TS (2012) Regulation of innate immunity by NADPH oxidase. Free Radic Biol Med 53:72–80
48. Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4:181–189
49. Taylor RM, Baniulis D, Burritt JB, Gripentrog JM, Lord CI, Riesselman MH, Maaty WS, Bothner BP, Angel TE, Dratz EA, Linton GF, Malech HL, Jesaitis AJ (2006) Analysis of human phagocyte flavocytochrome b_{558} by mass spectrometry. J Biol Chem 281:3745–3756
50. Gorudko IV, Mukhortava AV, Caraher B, Ren M, Cherenkevich SN, Kelly GM, Timoshenko AV (2011) Lectin-induced activation of plasma membrane NADPH oxidase in cholesterol-depleted human neutrophils. Arch Biochem Biophys 516:173–181
51. Gabius HJ, Gabius S (1999) Immunomodulierende Misteltherapie durch Lektinstandardisierung: Ein zweischneidiges Schwert? Versicherungsmedizin 51:128–136
52. Timoshenko AV, Lan Y, Gabius HJ, Lala PK (2001) Immunotherapy of C3H/HeJ mammary adenocarcinoma with interleukin-2, mistletoe lectin or their combination: effects on tumor growth, capillary leakage and nitric oxide (NO) production. Eur J Cancer 37:1910–1920
53. Holopainen T, Bry M, Alitalo K, Saaristo A (2011) Perspectives on lymphangiogenesis and angiogenesis in cancer. J Surg Oncol 103:484–488
54. Timoshenko AV, Chakraborty C, Wagner GF, Lala PK (2006) COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. Br J Cancer 94:1154–1163
55. Timoshenko AV, Kaltner H, André S, Gabius HJ, Lala PK (2010) Differential stimulation of VEGF-C production by adhesion/growth-regulatory galectins and plant lectins in human breast cancer cells. Anticancer Res 30:4829–4833
56. Brkovic A, Sirois MG (2007) Vascular permeability induced by VEGF family members in vivo: role of endogenous PAF and NO synthesis. J Cell Biochem 100:727–737
57. Timoshenko AV, Rastogi S, Lala PK (2007) Migration-promoting role of VEGF-C and VEGF-C binding receptors in human breast cancer cells. Br J Cancer 97:1090–1098
58. Kunze E, Schulz H, Gabius HJ (1998) Inability of galactoside-specific mistletoe lectin to inhibit *N*-methyl-*N*-nitrosourea-induced tumor development in the urinary bladder of rats and to mediate a local cellular immune response after long-term administration. J Cancer Res Clin Oncol 124:73–87
59. Kunze E, Schulz H, Adamek M, Gabius HJ (2000) Long-term administration of galactoside-specific mistletoe lectin in an animal model: no protection against *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder carcinogenesis in rats and no induction of a relevant local cellular immune response. J Cancer Res Clin Oncol 126:125–138
60. Xin X, Majumder M, Girish GV, Mohindra V, Maruyama T, Lala PK (2012) Targeting COX-2 and EP4 to control tumor growth, angiogenesis, lymphangiogenesis and metastasis to the lungs and lymph nodes in a breast cancer model. Lab Invest 92:1115–1128

Chapter 4
Bioactive Phytochemicals from Canadian Boreal Forest Species Used Traditionally by Eastern James Bay Cree Aboriginals to Treat Diabetes Mellitus

José A. Guerrero-Analco, Asim Muhammad, Ammar Saleem, Louis C. Martineau, Lina Musallam, Hoda M. Eid, Nan Shang, Paleah Black, Alain Currier, Pierre S. Haddad and John T. Arnason

Abstract Type-2 diabetes mellitus is a chronic metabolic disorder characterized by impaired insulin secretion and sensitivity, and is more pronounced among some indigenous populations due to their transition from traditional to modern diets, as well as their cultural disconnection from modern pharmacological treatment regimes. This is the case for the Cree Nations of Eeyou Istchee (CEI) of northern Quebec, where the age-adjusted prevalence of disease reached 29 %. The Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines is a multidisciplinary team aimed at alleviating the impact of this disease in the CEI by using a culturally adapted approach. A quantitative ethnobotanical study of traditional medicines conducted by our team in collaboration with Healers and Elders resulted in the identification of several potential antidiabetic plants. The crude extracts of these plants were tested in a comprehensive platform of in vitro bioassays designed to detect potential antidiabetic biological activities including: stimulation of glucose uptake in C2C12 muscle cells and potentiation of differentiation of 3T3-L1

J. T. Arnason (✉) · J. A. Guerrero-Analco · A. Muhammad · A. Saleem · P. Black
Centre for Advanced Research in Environmental Genomics,
University of Ottawa, Ottawa, ON K1N6N5, Canada
e-mail: jarnason@uottawa.ca

L. C. Martineau · L. Musallam · H. M. Eid · N. Shang · P. S. Haddad
Department of Pharmacology, Université de Montréal, Montréal, QC H3T 1J4, Canada

J. A. Guerrero-Analco · A. Muhammad · A. Saleem · L. C. Martineau · L. Musallam · H. M. Eid · N. Shang · A. Currier · P. S. Haddad · J. T. Arnason
Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines,
Montréal, QC H3T 1J4, Canada

L. C. Martineau · L. Musallam · H. M. Eid · N. Shang · P. S. Haddad · J. T. Arnason
Montreal Diabetes Research Centre, Université de Montréal,
Montréal, QC H3T 1J4, Canada

A. Currier
Institut de recherche en biologie végétale, Jardin botanique de Montréal,
Université de Montréal, Montréal, QC H1X 2B2, Canada

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_4

pre-adipocytes indicating enhanced insulin sensitivity. These procedures allowed us to identify the most significant species from the biological activity viewpoint, and they were considered for further phytochemical characterization. The present report provides a comprehensive summary of the major biological activities and phytochemistry of these key Canadian boreal forest plants that demonstrated significant ethnobotanical evidence of antidiabetic activity and associated symptoms.

Keywords Bioassay-guided fractionation · Canadian Boreal forest · Adipogenesis · Glucose uptake · Diabetes · Triterpenoids · Diterpenoids · Stilbenoids · Flavonoids · Lignans · Phenolics

Acronyms

NMR	Nuclear magnetic resonance spectroscopy
COSY	Homonuclear correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
NOESY	Nuclear Overhauser effect spectroscopy
HMQC	Heteronuclear multiple-quantum correlation spectroscopy
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HRMS	High resolution mass spectrometry
UV	Ultraviolet spectroscopy
IR	Infrared spectroscopy

4.1 Introduction

Type 2 or non-insulin-dependent diabetes mellitus (NIDDM) is a chronic metabolic disorder characterized by impaired insulin secretion and sensitivity. NIDDM has become a worldwide epidemic according to the World Health Organization (WHO) [1–4] and is more pronounced among indigenous populations due to their transition from traditional to modern diets, to a certain level of genetic predisposition and to their cultural disconnection from modern pharmacological treatment regimes. This is the case for the Cree Nations of Eeyou Istchee (CEI) of northern Quebec (Eastern James Bay Cree), where the age-adjusted prevalence of disease reached 29% in 2009 [5]. The burden of NIDDM and the ensuing deleterious complications (cardiovascular, retinopathy and nephropathy) prompted the search for culturally adapted complementary and alternative treatment options for these Aboriginal populations.

The CIHR Team in Aboriginal Antidiabetic Medicines is a multidisciplinary team aimed at alleviating the impact of this disease by using a culturally adapted approach. A quantitative ethnobotanical study of traditional medicines conducted

by our team in collaboration with Healers and Elders of six CEI communities resulted in the identification of several potential antidiabetic plants [6, 9, 10]. The crude drugs of 17 of these species were found to be most promising, including: *Abies balsamea* (L.) Mill. (Pinaceae), bark; *Alnus incana* subsp. rugosa (Du Roi) R.T. Clausen (Betulaceae), bark; *Gaultheria hispidula* L. Muhl. (Ericaceae), fruits; *Juniperus communis* L. (Cupressaceae), bark; *Kalmia angustifolia* L. (Ericaceae), leaves; *Larix laricina* K. Koch (Pinaceae), bark; *Lycopodium clavatum* L. (Lycopodiaceae), shoots; *Picea glauca* (Moench) Voss. (Pinaceae), bark; *Picea mariana* (Mill.) BSP (Pinaceae), cones; *Pinus banksiana* Lamb (Pinaceae), cones; *Populus balsamifera* L. (Salicaceae), bark, *Rhododendron groenlandicum* (Oeder) Kron and Judd (Ericaceae), leaves; *Rhododendron tomentosum* (Stokes) Harmaja (Ericaceae), leaves; *Salix planifolia* Pursh (Salicaceae), bark; *Sarracenia purpurea* L. (Sarraceniaceae), whole plant; *Sorbus decora* C.K. Schneid. (Rosaceae), bark; and *Vaccinium vitis-idaea* L. (Ericaceae), fruits. These Canadian boreal plants were identified as herbal remedies effective for the treatment of diabetic symptoms such as frequent urination, blurry vision and slow-healing wounds [6]. Plant samples of the above 17 plant species were collected with the guide and consent of local Cree Elders in CEI territory. The collection and identification of the plant species was done by Dr. Alain Cuerrier of the Montreal Botanical Garden, and voucher specimens were archived in the Marie-Victorin Herbarium™ of the Montreal Botanical Garden in Montreal, Québec, Canada.

The crude extracts of these plants were further tested in a comprehensive platform of in vitro bioassays designed to detect potential antidiabetic biological activities including: stimulation of glucose uptake in C2C12 muscle cells and potentiation of differentiation of 3T3-L1 pre-adipocytes indicating enhanced insulin sensitivity [7–9]. These procedures allowed us to identify the most significant species from the biological activity viewpoint and were considered for a further phytochemical characterization. The selected species include: *Alnus incana* subsp. *rugosa, Larix laricina, Populus balsamifera, Rhododendron groenlandicum, Rhododendron tomentosum, Sarracenia purpurea, Sorbus decora* and *Vaccinium vitis-idaea* [7–10]. The present report provides a comprehensive summary of the major phytochemical findings concerning key Canadian boreal forest plants that demonstrated significant ethnobotanical and biological evidence of antidiabetic activity. Nevertheless, additional experimental work is ongoing using in vivo diabetes models in order to provide more supporting information about the efficacy and potential mode of actions of these valuable resources and their derivatives. On the other hand, no toxicity has been reported by the traditional Healers with these particular traditional medicines, although they have provided caution about other species. No toxicity has been observed in our in vivo trials with these products (data not shown). Eventually, more formal toxicity evaluation is recommended, such as a 28-day feeding study at 10*x* bioactive dose. In the following sections, the methods used in the project are described as well as a summary of results for the key Cree medicinal plants..

4.2 Materials and Methods

4.2.1 Phytochemistry

Isolation of the putative antidiabetic principles responsible for the observed biological activities was carried out in all cases by a bioassay-guided fractionation approach using conventional phytochemical separation techniques such as open glass column (CC), thin layer (TLC) and high performance liquid (HPLC) chromatographies. Structural elucidation of all the new chemical entities was undertaken by spectroscopic and spectrometric methods including 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HMQC, HMBC) NMR, HRMS, UV and IR. Known compounds were identified by comparison of their physicochemical properties with those previously reported in the literature.

4.2.2 Glucose Uptake Assay

2-Deoxy-D-glucose uptake assay was performed as described previously with a few modifications.[8] Confluent and differentiated C2C12 myotubes (in 12-well plates) were incubated for 18 h in differentiation medium (DMEM with 2% horse serum) containing different concentrations of the crude extract, the fractions (100 μg/mL), or the pure compounds (6.25, 12.5, 25, 50 and 100 μM). Dimethyl sulfoxide (DMSO) (0.1%) and metformin (400 μM) were used as negative and positive controls, respectively. After 18 h, cells were washed twice with prewarmed (37 °C) Krebs phosphate buffer, pH 7.4 (136 mM NaCl, 20 mM HEPES, 4.7 mM KCl, 1 mM MgSO4, 1 mM $CaCl_2$, 4.05 mM Na_2HPO_4 and 0.95 mM NaH_2PO_4), containing glucose (5 mM) and then incubated in the same buffer for 30 min at 37 °C. After 30 min, cells were rinsed three times with Krebs phosphate buffer, pH 7.4 (37 °C), with no glucose. Glucose uptake was initiated by the addition of 500 μL (in each well) of Krebs phosphate buffer (37 °C) containing 10 μM 2-deoxy-D-glucose and 1 μCi/mL of [^{3}H-]-2-deoxy-D-glucose. After 10 min incubation at 37 °C, cells were rinsed three times with cold Krebs phosphate buffer (4 °C) containing glucose (5 mM), then lysed by incubating the samples with 500 μL of 0.1 M NaOH for 30 min at room temperature. Lysates were added to 4 mL of liquid scintillation cocktail (Ready-gel, Beckman Coulter Inc.), and radioactivity from [^{3}H]-2-deoxy-D-glucose incorporated into cells was measured in a scintillation counter.

4.2.3 Adipogenesis Assay

Pre-adipocyte 3T3-L1 cells were grown to confluency in Dubelcco's modified eagle medium (DMEM) containing 10% of bovine calf serum (BCS, proliferation medium). Twenty-four hour post-confluence (day 0), cells were induced to differentiate with

high-glucose DMEM supplemented with 10% of fetal bovine serum (FBS), 1 µM dexamethasone, 250 µM 3-isobutyl-1-methylxanthine (IBMX) and 500 nM insulin. After 48 h, the medium was replaced with DMEM containing 10% FBS and 500 nM insulin. Cells were differentiated for a total of 7 days with media changes every 2 days. Plant extract and fractions (25 µg/ml), compounds (0.5–100 µM) as well as rosiglitazone (10 µM; inducer of differentiation) were dissolved in (DMSO, 0.1% final concentration), added to the cells at day 0 of differentiation and replenished at every medium change thereafter.

Adipogenesis was assessed in the well-characterized 3T3-L1 cell model by measuring the accumulation of triglycerides (TG) upon differentiation (at day 7), using the AdipoRed reagent according to the manufacturer's instructions as described previously with minor modifications [8–9]. Briefly, after washing each well twice with Phosphate-Buffered Saline (PBS: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.68 mM KCl; pH 7.4), 2 mL of PBS containing 60 µl of AdipoRed reagent were added to each well and incubated for 15 min at room temperature. AdipoRed becomes fluorescent when partitioned in a hydrophobic compartment. The fluorescence of each well was measured by excitation at 485 nm and emission at 572 nm. The results were reported as percentage of the vehicle control (0.1% DMSO). The compounds were tested at varying concentrations ranging from 0.5 to 25 µg/ml (0.5–100 µM) on 3T3-L1 cells to determine their adipogenic effect.

4.2.4 Statistical Analysis

Intracellular TG and [^{3}H]-2-deoxy-D-glucose content results are presented as mean ± SEM of three independent experiments, each performed in triplicate. Statistical calculations and EC_{50} (calculated using sigmoidal dose–response (variable slope)) analysis was performed with Prism GraphPad software. Differences between groups were analysed by one-way analysis of variance (ANOVA). A p value below 0.05 was considered statistically significant.

4.3 *Sorbus decora* (Rosaceae)

S. decora, commonly known as showy mountain ash, is a widely distributed tree of the boreal forest of North America [11]. The plant is known as muskuminanatikw. Traditionally, the bark is used by the CEI to treat symptoms associated with diabetes mellitus as well as other diseases like muscle and back aches, colds, rheumatism and coughs [6, 8–10, 12–13]. We have described the first comprehensive phytochemical investigation from an active ethanol (EtOH) (80% in water) extract via a bioassay-guided separation based on in vitro glucose uptake by C2C12 cells and the activity of various fractions as well as the isolated pure components [14]. This effort has resulted in the isolation and structure elucidation of three new

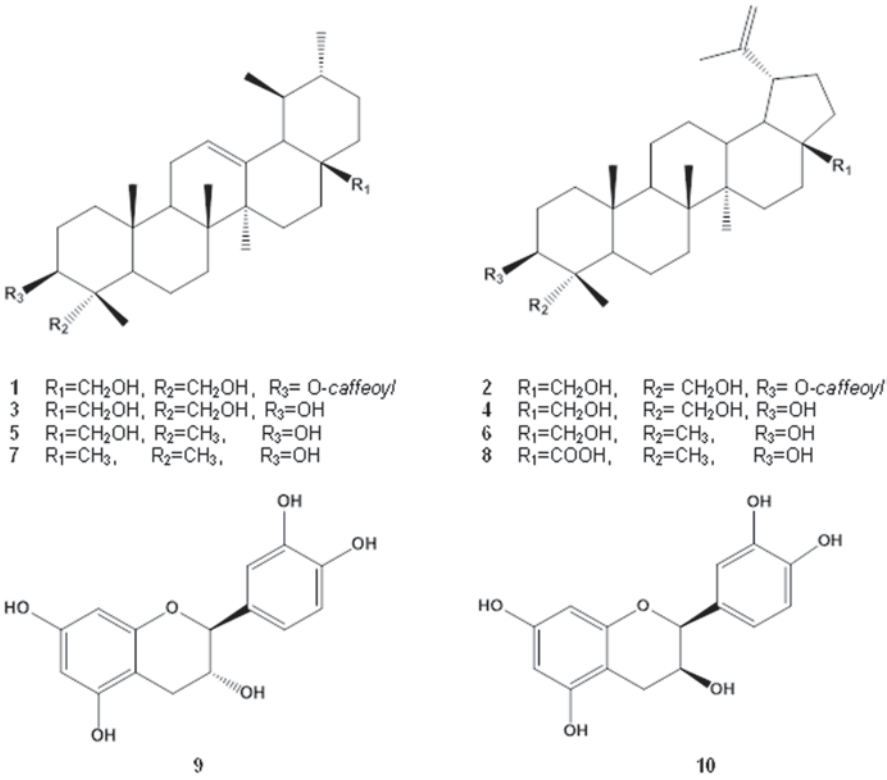


Fig. 4.1 Compounds isolated from the stem bark of *S. decora*

triterpenoids: 23,28-dihydroxy-urs-12-ene-3*β*-caffeate (1), 23,28-dihydroxy-lup-12-ene-3*β*-caffeate (2), and 3*β*-23,28-trihydroxyurs-12-ene (3). In addition, seven known compounds were also isolated from the active extract, including 23-hydroxy-betulin (4), uvaol (5), betulin (6), α-amyrin (7) and betulinic acid (8), (+)-catechin (9) and (−)-epicatechin (10) [14]. The chemical structures of compounds 1–10 are shown in Fig. 4.1. One of the new triterpenes, compound 2, showed significant antidiabetic properties as determined by an increase in glucose uptake by the C2C12 cells when compared with the positive control metformin (400 μM). The bioactivity results were concentration dependent when cells were treated with active extracts or purified active principle. The EC_{50} values estimated using linear regression of the uptake versus log *C* (concentration) varied from 1.28 to 25.5 μg/mL [14]. The bioactivity results (Fig. 4.2) showed that both crude extract (SDE) and compound 2 exhibited potent glucose uptake activity, suggesting that 2 underlies, at least in part, the antidiabetic effects observed with the crude extract in vivo experiments where *S. decora* demonstrates both anti-hyperglycemic and insulin-sensitizing activity in mice [15]. Further work is needed to confirm the mode of action of 2.

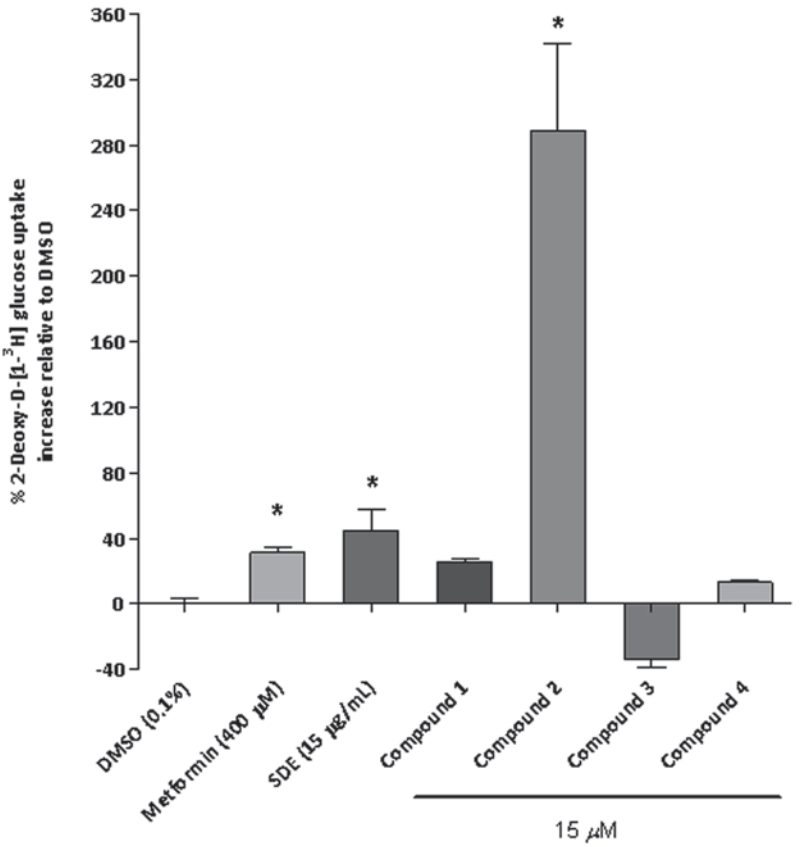


Fig. 4.2 Effect of the crude extract (SDE) of *S. decora* at the maximum concentration (15 µg/mL) and pure compounds (1–4, 15 µM) tested on glucose uptake assay in C2C12 cells. Each value is the mean ± SE ($n = 4$ wells) in each group. *$p < 0.05$ Significantly different after one-way ANOVA analysis followed by Dunnett's *t*-test versus the negative control group (DMSO 0.1%). (Adapted with permission from [14]. Copyright 2010 American Chemical Society)

4.4 *Larix laricina* (Pinaceae)

L. laricina, commonly known as watnagan (Cree), tamarack (English) or mélèze (French), is a highly regarded plant used by many First Nations throughout Canada as a culturally important medicine to treat several ailments, including cuts, burns, coughs, stomach pain, sores (mouth, skin and throat), heart problems and frostbite [12, 13,16–19]. The bark was especially identified for the treatment of diabetic sores, frequent urination, poultice on infected wounds and the folk description "to heal the insides" by Aboriginals of Eastern Canada [16, 20–21]. It was notably found to significantly enhance adipogenesis in differentiating 3T3-L1 adipocytes in a screening study using in vitro cell-based bioassays [8]. We used the same cell line to carry out a bioassay-guided fractionation of an 80% EtOH extract (LLE) in

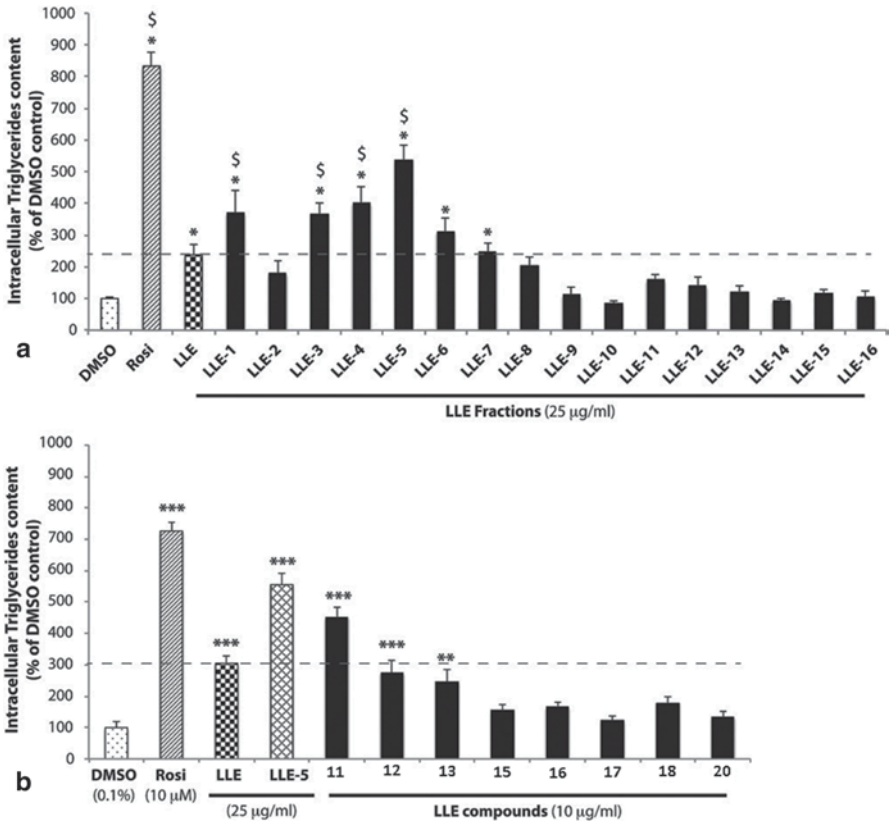


Fig. 4.3 The adipogenic activity of *L. laricina* crude extract (LLE; *dotted line* added for ease of comparison), fractions (LLE-1–LLE-16) at 25 µg/mL and compounds. **a** Preadipocytes (3T3-L1 cells) were induced to differentiate in the presence of the crude extract and fractions or rosiglitazone (Rosi, inducer of differentiation; 10 µM). 0.1% DMSO was used as vehicle control. Following 7 days of differentiation, cells were washed and the intracellular content of triglycerides (TG) was determined. *Denotes statistically significant from vehicle control ($p \leq 0.05$). $ Denotes statistically significant from crude extract LLE ($p \leq 0.05$). **b** Preadipocytes were treated with rosiglitazone (10 µM), LLE (25 µg/ml), LLE-5 (25 µg/ml) and eight of the isolated compounds (10 µg/ml) for 7 days then TG content was determined. **Denotes statistically significant from vehicle control ($p \leq 0.01$). ***Denotes statistically significant from vehicle control. ($p \leq 0.001$; adapted with permission from [22])

order to identify potential active principles of the plant. The results from a silica gel column chromatography (CC) showed that, from a total of 16 primary fractions (LLE-1 to LLE-16), only LLE-1, LLE-3, LLE-4 and LLE-5 exhibited the largest increases in 3T3-L1 cell adipogenesis (Fig. 4.3) [22], the most active one (LLE-5) reaching more than double the activity of the crude LLE and some two-thirds of the activity of the reference drug rosiglitazone. Ten compounds (Fig. 4.4) were further isolated by preparative reversed phase HPLC (30 min linear gradient of 20–80% of CH_3CN in water at the flow rate of 31.5 ml/min) from the active

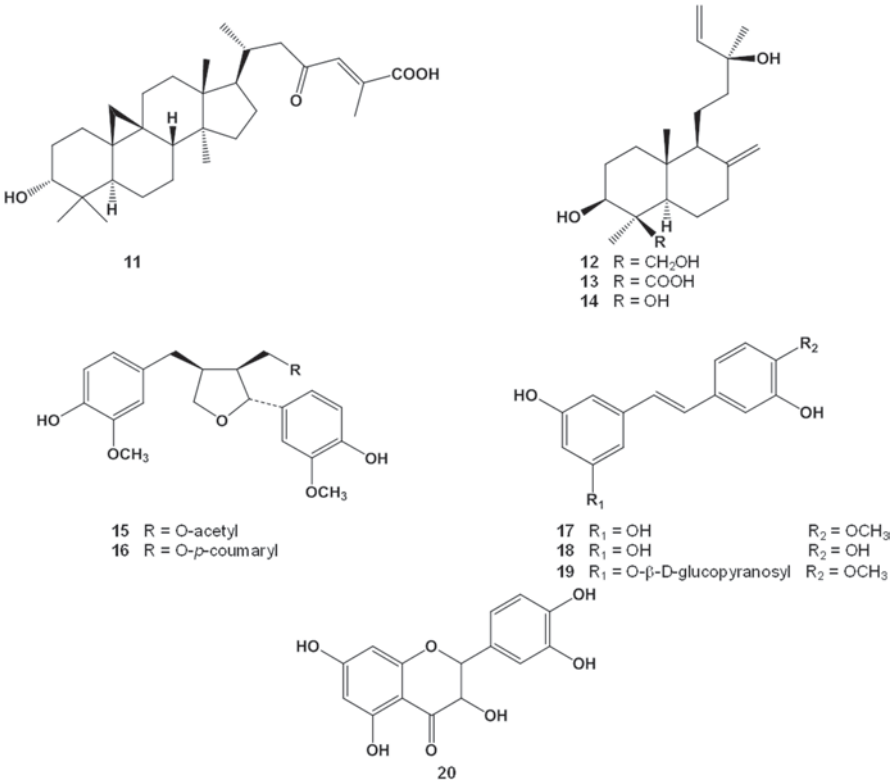


Fig. 4.4 Compounds isolated from the bark of *L. laricina*

fractions, and two compounds (11 and 12) were found to be most active, displaying physiological dose-response curves (data not shown) [22]. We identified one major active principle responsible for the effect of *Larix laricina* on adipogenesis [22]. Compound 11 is a previously unidentified cycloartane triterpenoid, 23-oxo-3α-hydroxycycloart-24-en-26-oic acid, which strongly potentiates adipogenesis with an EC_{50} of 7.7 μM. In addition, we reported a new biological activity for 13-epitorulosol (12, EC_{50} 8.2 μM) and rhapontigenin (17, EC_{50} 127 μM) at high concentrations. Although compounds 12 and 17 had previously been identified in *L. laricina* [23], this is the first report of such antidiabetic activity. Indeed, there is currently no reported biological activity for compound 12. On the other hand, compound 17, a natural analog of resveratrol with known antioxidant and anticancer activities, has been shown to inhibit mammalian intestinal-glucosidase activity (responsible for the first step in glucose degradation and therefore absorption) [24]. Further investigation is needed to determine the possible synergistic activity between compound 13 (13-epicupressic acid), compound 15 (lariciresinyl-3-acetate) and compound 16 (lariciresinyl-9′-*p*-coumarate) at the concentrations present in the most active fraction [22]. On the other hand, no antidiabetic activity was found for

compounds 14 (19-norlabda-8(17),14-diene-4,13-diol), 18 (piceatannol), 19 (rhapontecin) and 20 (taxifolin) in our study. Finally, ours is the first study that focuses on the identification of the active principles responsible for the adipogenic effect as the putative antidiabetic mechanism of *L. laricina*, as used by traditional Healers in Canadian Aboriginal populations. In support of this, we have recently shown that *L. laricina* decreases hyperglycemia and insulin resistance in vivo, using the diet-induced obese mouse model [25].

4.5 *Sarracenia purpurea* (Sarraceniaceae)

S. purpurea, commonly known as ayigadash (Cree) or purple pitcher plant (English), was among the top-ranked species in the syndromic importance value for diabetes symptoms. It is a carnivorous plant, which is widely distributed across North America [26]. These plants adapt to their nitrogen-poor habitats (such as bogs and peatlands) by consuming nitrogen from insects trapped within their pitchers (fused leaves). The purple pitcher plant is well known for its medicinal properties and was mentioned in the treatment of urination problems, headaches, lower back pain, fever and dysmenorrhea [6,12,18]. *S. purpurea* extract exhibited interesting antidiabetic potential in an initial screening study. In particular, *S. purpurea* extract significantly increased glucose uptake in C2C12 mouse muscle cells under basal and insulin-stimulated conditions [8]. This plant extract also decreased hepatic glucose output by reducing the activity of glucose-6-phosphatase, a key enzyme in gluconeogenesis, and by increasing glucose storage through an increase in glycogen synthase activity (Nachar, Haddad et al., unpublished observation). In addition, the extract also protected PC12 neuronal cells against cell death caused by hyper- or hypoglycemic conditions [8]. Therefore, this plant exhibits both primary (glycemia-lowering) and secondary (protection against diabetes complications) antidiabetic activities. Given these interesting antidiabetic effects, a bioassay-guided fractionation approach was taken by our team, with the main goal of identifying the active principles of *S. purpurea* responsible for modulating muscle glucose uptake.

The dried leaves of *S. purpurea* were extracted with 80% aqueous EtOH and fractionated using polyamide-6 chromatography. Separation of the EtOH extract (SE) resulted in eight fractions (SE-1 to SE-8). The crude extract and fractions SE-3 to SE-7 (100 μg/mL) significantly increased glucose uptake in C2C12 mouse muscle cells between 30 and 60% as compared to a vehicle control (Fig. 4.5). Further separation of the active fractions by preparative recycling reversed-phase HPLC (flow rate of 5 ml/min with 60% H_2O in MeCN) resulted in the identification of the new 6′-*O*-caffeoylgoodyeroside (21) and ten known compounds (Fig. 4.6): taxifolin-3-*O*-glucoside (22), 7α-*O*-methylmorroniside (23), 7β-*O*-methylmorroniside (24), rutin (25), isorhamnetin-3-*O*-glucoside (26), kaempferol-3-*O*-rutinoside (27), kaempferol-3-*O*-(6″-caffeoylglucoside) (28), morroniside (29), goodyeroside (30) and quercetin-3-*O*-galactoside (31). Pure compounds were initially tested at 50 μM to determine their effect on glucose uptake in C2C12 muscle cells. Most isolates

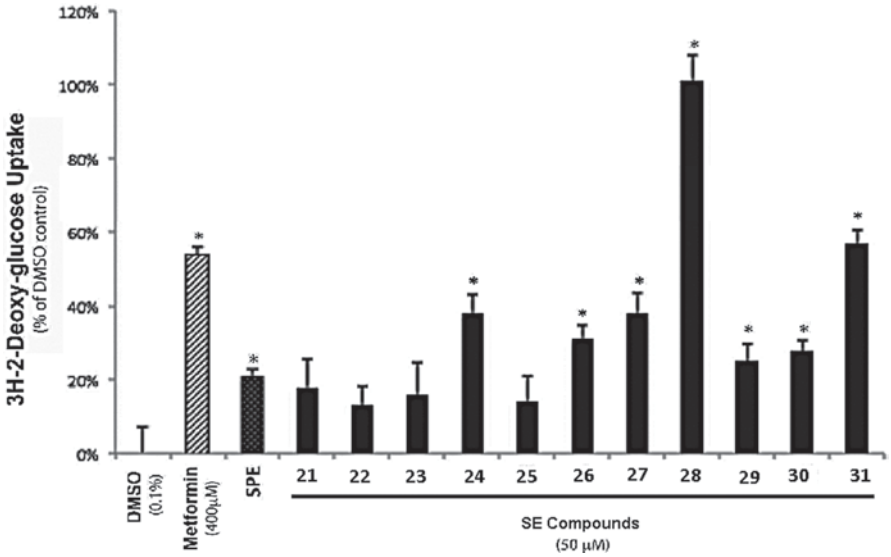


Fig. 4.5 Effect of the crude extract (SE; 100 μg/mL) and isolated compounds (21–31; 50 μM) of *S. purpurea* on glucose uptake in C2C12 mouse muscle cells. Results are presented as mean ± SE ($n=4$ wells) for each group. *Denotes significantly different as compared to DMSO 0.1% control ($p<0.05$) using one-way ANOVA analysis followed by Dunnett's *t*-test. (Adapted with permission from [24]. Copyright 2012 American Chemical Society)

(except isolates 21−23 and 25) showed significant potentiation of glucose uptake (ranging between 25 and 101%) as compared to vehicle control ($p \leq 0.05$, Fig. 4.5). We then acquired a dose-response curve for each of the seven promising isolates at concentrations ranging from 6.25 to 100 μM in order to determine their EC_{50} value [27]. Compounds 26, 28 and 31 exhibited significant potency and the highest maximal efficacy on glucose uptake, suggesting that these compounds are most likely the active principles responsible for the ability of *S. purpurea* to potentiate glucose uptake in these cells. It is noteworthy that 31 (quercetin-3-*O*-galactoside), which significantly potentiated glucose uptake in C2C12 muscle cells in this study ($EC_{50}=60.5$ μM; max = 211%; Fig. 4.5), has previously been identified as one of the active principles of *Vaccinium vitis-idaea* berries [28]. Kaempferol and its derivatives, on the other hand, were reported to have mixed effects on glucose uptake. Indeed, kaempferol and kaempferol-3-neohesperidoside have been shown to stimulate glucose uptake in adipocytes and muscle cells, respectively [29, 30]. On the other hand, kaempferitine inhibited glucose uptake in adipocytes. Kaempferol-3-*O*-(6″-caffeoylglucoside), identified herein as compound 28, is a potent stimulator of glucose uptake, with an EC_{50} value of 13.8 μM and maximal efficacy of 188% as compared to vehicle control. Finally, compound 26 also stimulated glucose uptake in C2C12 muscle cells ($EC_{50}=18.5$ μM; max = 150% of vehicle control; Fig. 4.5), thereby providing the first evidence for a potential direct glycemia-lowering effect for this compound [27].

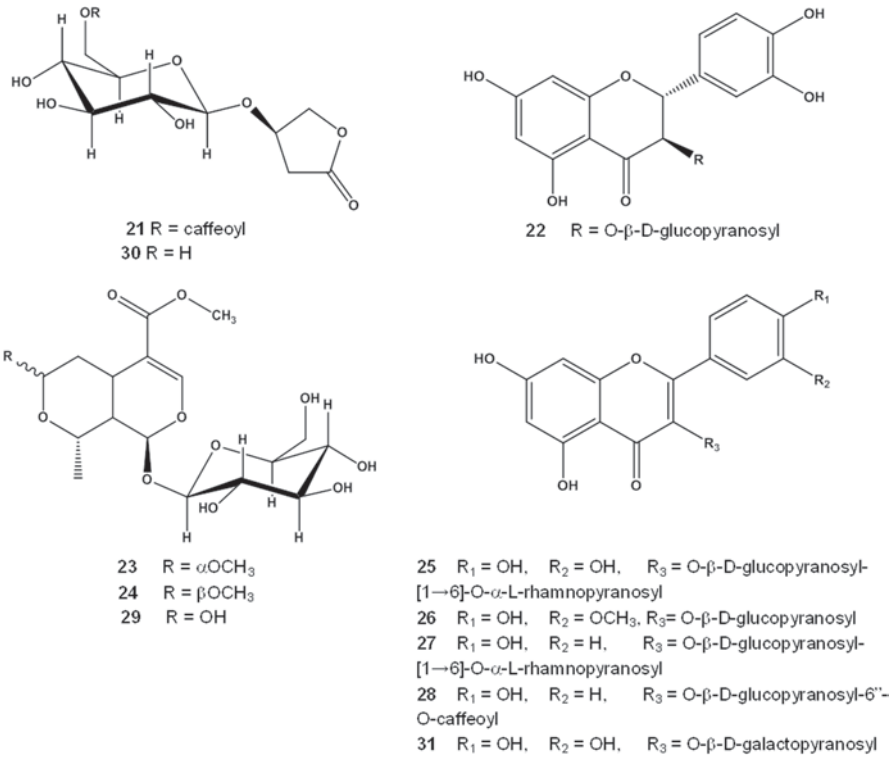


Fig. 4.6 Compounds isolated from the leaves of *S. purpurea*

4.6 *Alnus incana* subsp. *rugosa* (Betulaceae)

Alnus (atushpi in Cree) bark is widely used for the treatment of skin conditions and diabetic sores [20] and was among the top eight antidiabetic treatments in the Cree territories [6]. Within the Cree Nations of Canada, a number of uses have been mentioned in the literature: coughs, sore throat, mouth sores [13, 17], sore eyes and laxative [19]. In the course of screening experiments on antidiabetic potential using 3T3-L1 adipocyte cell model to assess glitazone-like stimulation of adipogenesis, the EtOH (80% in water) extract of *A. incana* subsp. *rugosa* was found to be very active. However, it exerted an inhibitory effect, suggesting a potential antiobesity action. Therefore, a classical activity-guided fractionation approach was used to isolate its constituents with anti-adipogenic activity. The process was guided by the assessment of triglyceride content in cells treated with fractions or isolates. The phytochemical and bioactivity results are summarized in the scheme shown in Fig. 4.7, with anti-adipogenic activity (% inhibition) indicated under each fraction or isolate. Fractionation of *Alnus* extract was initially performed using various solvents. The hexane extract was found to be inactive. The hexane-insoluble material was extracted in ethyl acetate, and this extract was found to be more active than the

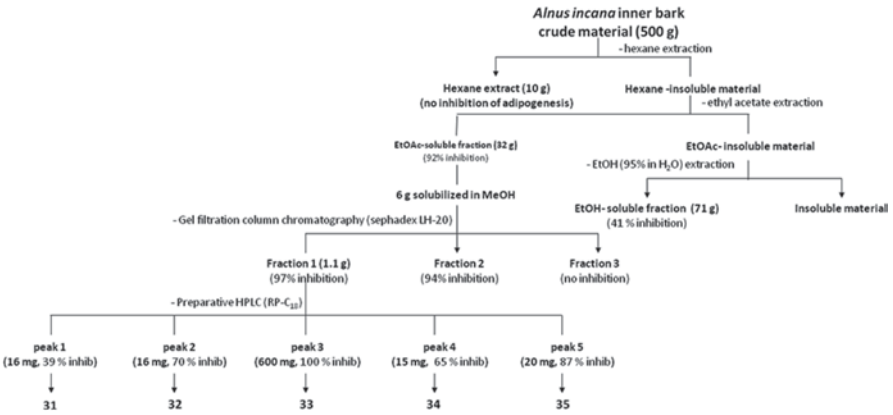


Fig. 4.7 Bioassay-guided fractionation of the extract of *A. incana* subsp. *rugosa* was performed according to this scheme and resulted in the isolation and identification of oregonin (33) as the active principle. At each level of fractionation, all fractions generated were tested simultaneously and were compared to the crude extract tested in parallel. All tests were performed at the same concentration used for the crude extract (50 µg/mL). Fractionation was guided by inhibition of adipogenesis. Mean inhibitory activity for $n=4$ wells of a single cell preparation is indicated in *brackets* under each fraction. Yields are indicated in g or mg beside each fraction. (Adapted with permission from [32])

crude extract. The ethyl acetate-insoluble material was then extracted in 80 % ethanol, but the resulting fraction was found to be less active than the crude extract. The ethyl acetate fraction was therefore further purified and subjected to gel filtration chromatography using water/methanol as solvents. Fractions were pooled according to analytical HPLC profiles, resulting in three distinct fractions, of which fraction 1 was found to be most active. This fraction was further purified by preparative HPLC to yield five compounds: rubranoside A (32), 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane-3-*O*-*β*-D-xylopyranoside (33), oregonin (34), alnuside A (35), alnuside B (36). All five compounds (Fig. 4.8) had previously been reported [31–34]. They were all tested at equal concentration, and only oregonin was found to be as active as the crude extract; the other four compounds exhibited various degrees of inhibitory activity, in every case inferior to the activity of the crude extract, despite more than an order of magnitude greater enrichment than oregonin [35].

4.7 *Populus balsamifera* (Salicaceae)

Although poplar buds are a well-known anti-inflammatory medicine, poplar bark is used as Spring food by the Cree and other Eastern Canadian First Nations [20]. Nonetheless, as far back as 1884, Holmes mentioned the use of bark as a purgative or antitussive medicine [16]. Other uses encompass the treatment of boils and abscesses, rashes, snow blindness [13, 17], nosebleed and sores [12], high blood pressure, child asthma, eczema and psoriasis, heart problems, teething toothache and diabetes [6, 19].

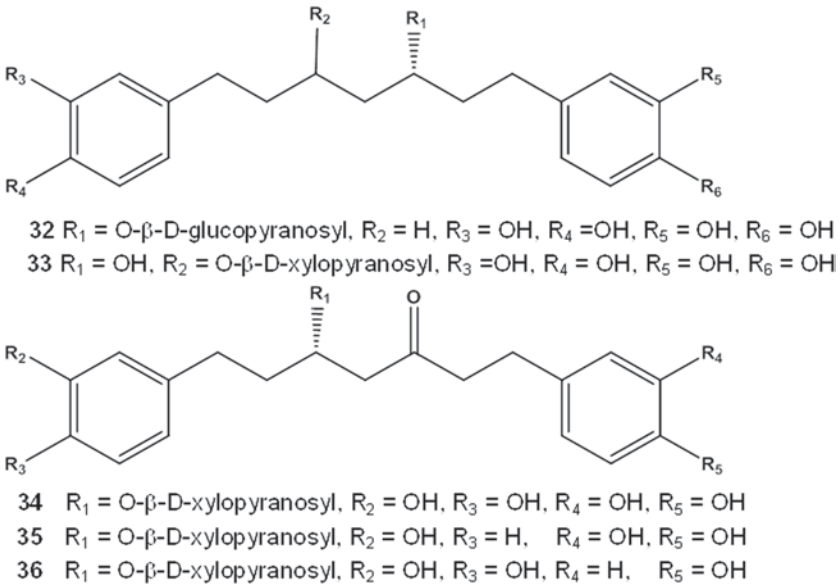


Fig. 4.8 Compounds isolated from the bark of *A. incana*

An activity-guided fractionation approach similar to that employed for *A. incana* was used to isolate bioactive constituents of *P. balsamifera* (mitush in Cree) bark extract. Indeed, the plant exhibited unexpected and even more pronounced anti-adipogenic activity. Moreover, antiproliferative activity was also observed and served as an additional bioactivity guide. Results are presented in Scheme 2 shown in Fig. 9, with % inhibitory activity data indicated under each fraction or isolate. Fractionation of *P. balsamifera* inner bark begun by extraction in 80% ethanol, and the crude extract was washed with hexane to remove waxy material. The hexane-insoluble material was dissolved in methanol and subjected to gel filtration chromatography. Elution and pooling of fractions, as carried out for *A. incana*, resulted in nine fractions, of which fractions 2 and 3 completely inhibited adipogenesis. Since fraction 2 exhibited better anti-proliferative activity, this fraction was subjected to gel filtration chromatography, and it yielded six subfractions, of which subfractions 2.3 and 2.4 exhibited complete anti-adipogenic activity. As both had similar composition, only 2.3 was further purified by preparative HPLC, as carried out for *A. incana*, yielding five subfractions, of which 2.3.3, 2.3.4, and 2.3.5 completely inhibited adipogenesis. Subfraction 2.3.1 was composed exclusively of salicin (37) [i.e. 2-(hydroxymethyl)-phenyl-β-D-glucopyranoside] and was almost completely inactive. Subfraction 2.3.5 was composed exclusively of salicortin A (38). Both salicin and salicortin (Fig. 10) are well-known compounds [36].

The three other subfractions were composed of two salicortin diastereomers (salicortin A and B (39)). Using subfraction 2.3.2 as starting material, the isomers were isolated, and both were found to induce complete inhibition of adipogenesis

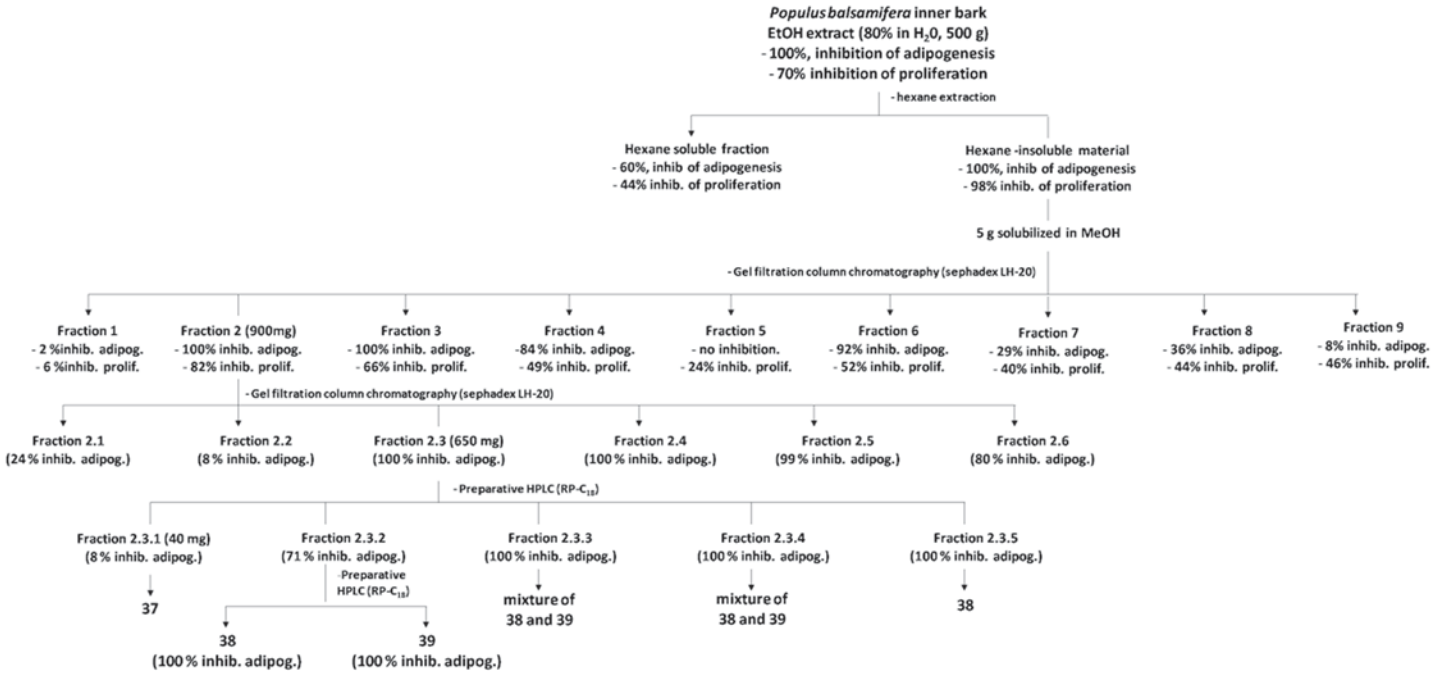


Fig. 4.9 Bioassay-guided fractionation of the extract of *P. balsamifera* was performed according to this scheme and resulted in the isolation and identification of salicortin isomers (38 and 39) as the active principles. At each level of fractionation, all fractions generated were tested simultaneously and were compared to the crude extract tested in parallel. All tests were performed at the same concentration used for the crude extract (100 μg/mL). Fractionation was guided by inhibition of adipogenesis and proliferation. Mean inhibitory activity for $n=4$ wells of a single cell preparation is indicated in *brackets* under each fraction. Yields are indicated in g or mg beside each fraction. (Adapted with permission from [32])

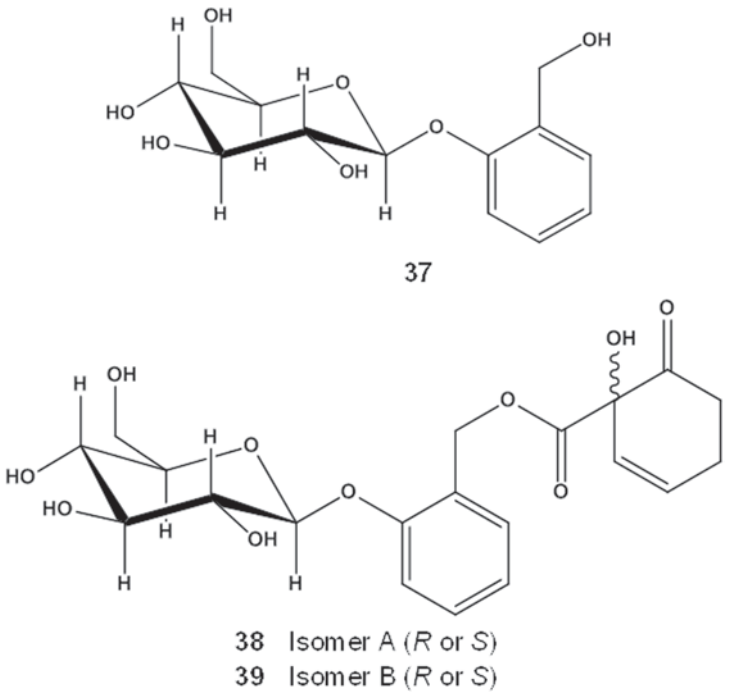


Fig. 4.10 Compounds isolated from the bark of *P. balsamifera*

[35]. Following isolation and identification, quantification of salicortin diastereomers A and B was performed using the crude extract. Together, these active principles accounted for 10% of the extract mass.

4.8 *Vaccinium vitis-idaea* (Ericaceae)

Various members of the *Vaccinium* genus, including lowbush blueberry (*V. angustifolium*), American cranberry (*V. macrocarpon*) and European bilberry (*V. myrtillus*), are traditionally used for the treatment of diabetes by several cultures around the globe [37]. *Vaccinium vitis-idaea* berry extract, known as wishichimna (Cree), was also found to enhance muscle cell glucose uptake in the antidiabetic screening studies already mentioned [9]. We thus sought to verify if these species exerted their effect by a mechanism similar to other boreal forest medicinal plant species [38,39], while simultaneously elucidating the active principles by using our expertise in the phytochemistry of Ericaceae [40–42].

In order to identify its active principles, the *V. vitis-idaea* berry extract was fractionated using a multistep approach guided by the enhancement of glucose uptake activity in C2C12 cells. All fractions were tested at 200 µg/mL and all

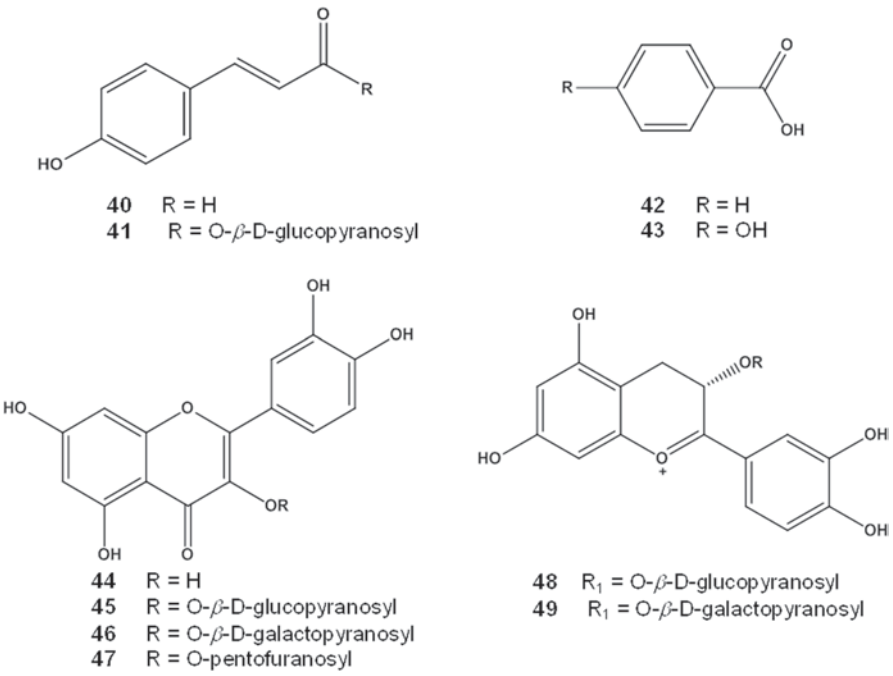


Fig. 4.11 Compounds isolated from the fruits of *V. vitis-idaea*

isolates at 100 µM. The crude ethanol extract was first fractionated into ethyl acetate-soluble and -insoluble fractions. Only the ethyl acetate-soluble fraction showed a significant stimulation of glucose uptake (41 ± 5 % above vehicle control) and was selected for further fractionation on a Sephadex LH-20 column. This yielded six subfractions pooled according to similar HPLC profiles. Of these, subfractions 2 and 3 showed significantly higher stimulation of glucose uptake than the original *V. vitis-idaea* berry extract and other fractions (377 ± 3 and 527 ± 4 %, respectively). These two fractions were selected for further fractionation. Using preparative HPLC chromatographic fractionation, five compounds were isolated from subfractions 2 and 3 (Fig. 4.11): *p*-coumaric acid (40), *p*-coumaroyl-D-glucose (41), benzoic acid (42), *p*-hydroxybenzoic acid (43) and quercetin (44). Of these, only quercetin stimulated uptake when tested at 100 µM (377 ± 9 %; $n = 6$). Finally, eight additional compounds (Fig. 4.11) were identified from subfraction 3: quercetin-3-*O*-glucoside (45), quercetin-3-*O*-galactoside (46), an unidentified quercetin-3-*O*-pentoside (47), (+)-catechin (9), (−)-epicatechin (10), cyanidin-glucoside (48) and cyanidin-galactoside (49) [27]. The first five of these compounds were isolated, and the three quercetin-3-*O*-glycosides were found to significantly enhance glucose uptake at 100 µM (597 ±, 387 ± 4 and 247 ± 3 %, respectively; $n = 6$). Cyanidin glycosides were also tested and found to be inactive [28].

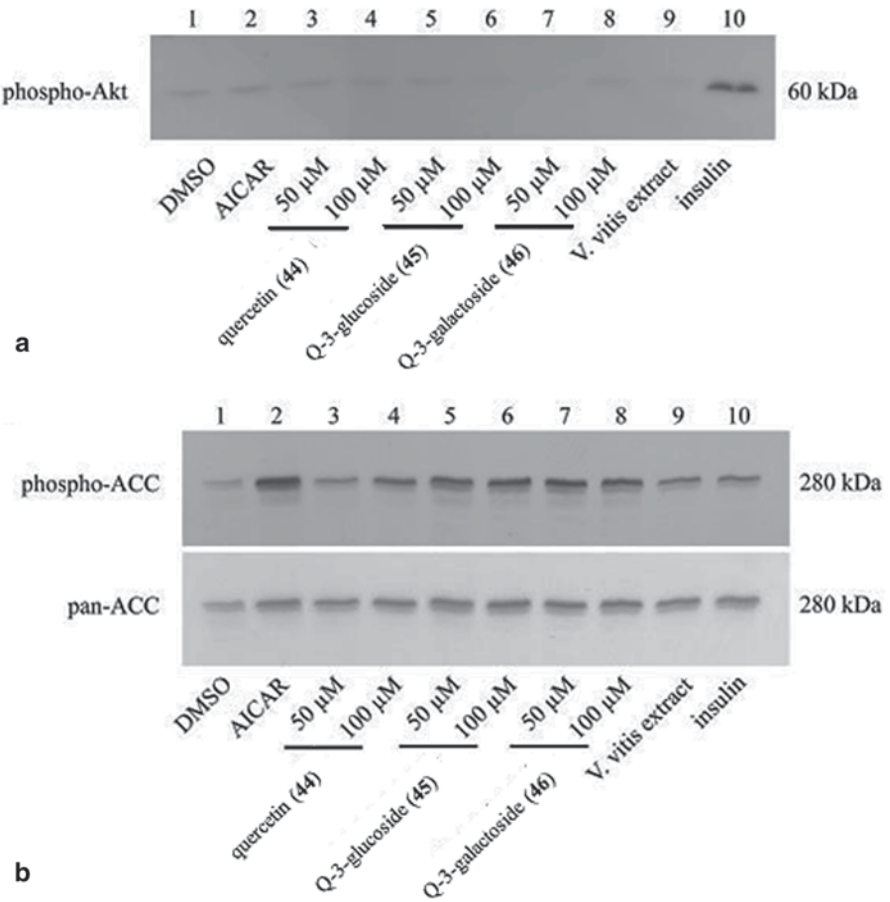


Fig. 4.12 *V. vitis-idaea* berry extract and its active principles stimulate the AMPK signalling pathway but not the insulin receptor pathway. C2C12 skeletal muscle cells were treated for 18 h with either 0.1 % DMSO (vehicle), 200 mg/ mL of *V. vitis-idaea* berry extract, or 50 and 100 µM of quercetin (44), quercetin-3-*O*-glucoside (45) or quercetin-3-*O*-galactoside (46). Phosphorylation of the insulin receptor pathway marker Akt (**a**) and of the AMPK effector or ACC (**b**) was measured by western immunoblot. Insulin (100 nM) and AICAR (2 mM) applied for 30 min served as positive controls. (Adapted with permission from [25])

To understand the mechanism mediating the effect of *V. vitis-idaea* berry extract on skeletal muscle cell glucose uptake, we evaluated the activity of the two main signalling pathways that regulate the rate of glucose uptake in this cell type: namely, the insulin-receptor pathway and the AMPK pathway. The effects of plant products on the insulin and AMPK signalling pathways of C2C12 muscle cells or H4IIE hepatocytes were assessed by western immunoblot. In C2C12 cells, there was no indication of increased phosphorylation of the protein kinase B (Akt/PKB, Fig. 4.12a), a marker of the former pathway, whereas insulin, as expected, produced a clear activation of this enzyme. Conversely, treatment of C2C12 with the

extract increased the phosphorylation of AMPK and the downstream acetyl-CoA carboxylase enzyme (ACC, Fig. 4.12b). AICAR, an AMP mimetic and known activator of AMPK signalling, served as a positive control and also greatly enhanced activation of ACC. Concordant with the activity of the crude extract, treatment of C2C12 cells for 18 h with 50 or 100 µM of compounds 44, 45 or 46 did not increase phosphorylation of Akt (Fig. 4.12a) but increased phosphorylation of ACC (Fig. 4.12b). Total content of ACC was not significantly altered by any treatment, indicating the modulation of enzyme activation rather than a change in protein expression.

AMPK is recognized as an important therapeutic target for diabetes [43–45]. Indeed, the effects of Metformin are mediated through this metabolic master enzyme and transducer of metabolic stress. Upon activation by an increase in the cellular ratio of AMP to ATP, AMPK serves to restore energy homeostasis by increasing flux through energy-producing pathways and by decreasing energy-consuming processes [46]. Energy production is increased by simultaneous enhancement of uptake and oxidation of lipids and carbohydrates. Some more tissue-specific effects include the insulin-like inhibition of hepatic glucose output and the translocation of GLUT-4 glucose transporters in skeletal muscle, activities that contribute to a systemic anti-hyperglycemic effect [47–50]. In addition to acute actions for restoring energy homeostasis, the activation of AMPK produces long-term adaptive effects, such as increased capacity for substrate uptake and oxidation that confer protection against future metabolic stresses [51–52].

Our studies thus suggest that quercetin and certain glycosides of this well-studied and widely distributed flavonoid [53] transiently inhibit mitochondrial ATP-synthase, leading to the activation of AMPK, and in addition that quercetin glycosides are responsible in part for the antidiabetic activity of *V. vitis-idaea* and perhaps of other species of this genus.

Finally, these results were further confirmed by our team using in vivo models for type 2 diabetes in KKAy and C57BL/6 mice [54]. In both models, *V. vitis-idaea* extract significantly decreased glycemia and strongly tended to decrease insulin levels, and this was accompanied by reduced fluid intake in the KKAy model. This correlated with either a tendency or a significant increase in GLUT4 content and activation of the AMPK and/or Akt pathways in skeletal muscle. *V. vitis-idaea* treatment also improved hepatic steatosis by decreasing hepatic triglyceride levels and significantly activating the AMPK and Akt pathways [54].

Finally, it is also known that quercetin and its glycosidic derivatives have been found to have poor bioavailability in mammals [55]. Without any doubt, further scientific work is needed to investigate which factors (physical, chemical and idiosyncratic) may be playing a key role in the improvement of such properties of these known biologically active compounds, as has also been described by several authors [55 and references there cited].

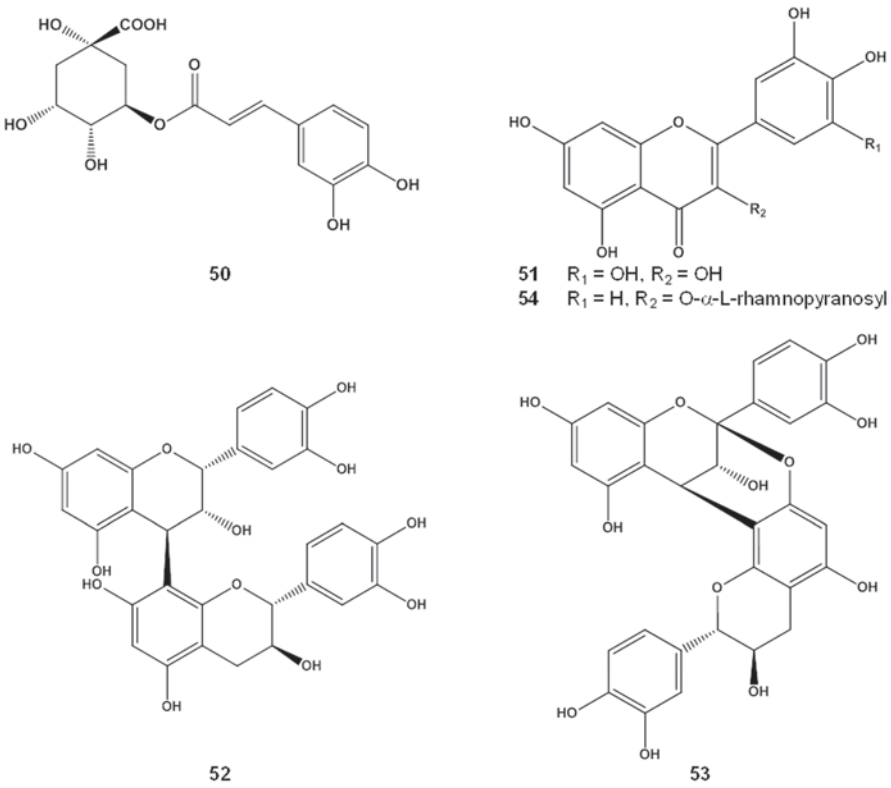


Fig. 4.13 Additional compounds isolated from *R. groenlandicum*

4.9 *Rhododendron groenlandicum* (Ericaceae)

R. groenlandicum, a plant of the heath family commonly known as kachichepukw (Cree) or Labrador tea (English), grows in the tundra, bogs and forests of North America [56]. It is highly recommended by many Cree Elders as a plant with strong antidiabetic potential [6, 57]. Among the Cree Nations of Canada, the plant is equally known to possess properties that can help in the healing of wounds [16], the treatment of coughs, cold, chills, infections (skin, urinary and other), upset stomachs, diarrhea, arthritis, backaches, headaches [13, 17–19], burns and scalds, [18, 57] as well as heart and kidney problems. There is also a mention in Beardsley (1941) [58] about Labrador tea being a possible emetic, while Marles et al. (2000) [19] recorded its use against bad breath, pneumonia, infants' teething pain and rashes and eye infections. In our screening studies, it stimulated glucose uptake in muscle cells, but more importantly induced adipogenesis as strongly as the reference drug rosiglitazone [8]. This would mean less free fatty acids circulating and accumulating in insulin-sensitive tissues such as the liver and the muscle. Such activity is

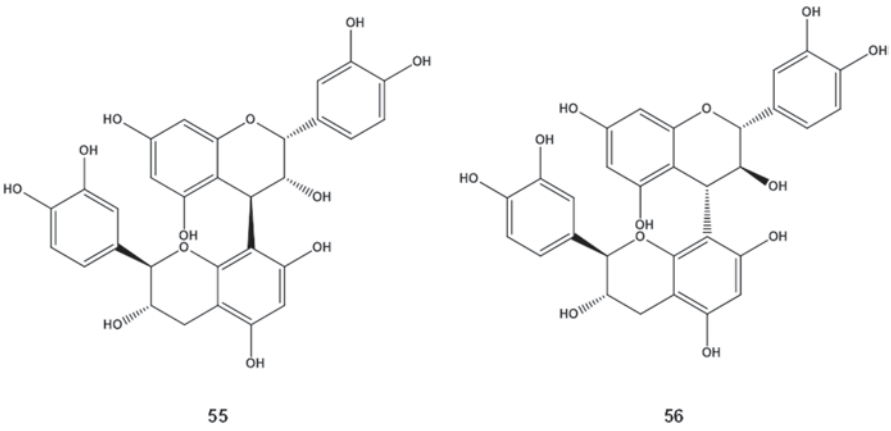


Fig. 4.14 Additional compounds isolated from *R. tomentosum*

also associated with an improvement of insulin sensitivity. Our team has previously reported the phytochemical characterization of Labrador tea crude ethanolic extract [59]. In that study, we identified (+)-catechin (9), (−)-epicatechin (10), chlorogenic acid (50), myricitin (51), procyanidin B2 (52), procyanidin A1 (53) and several quercetin glycosides (quercetin-3-*O*-glucoside (45), quercetin-3-*O*-galactoside (46); rutin (25) and quercetin-3-*O*-rhamnoside (54)). The additional chemical structures of the isolated compounds from *R. groenlandicum* are shown in Fig. 4.13. As mentioned, our previous screening studies revealed that *R. groenlandicum* strongly stimulates the differentiation of 3T3-L1 cells (adipogenesis) [8] and additional phytochemical work is currently being conducted by our team in order to identify the constituents responsible for the observed biological activity.

4.10 *Rhododendron tomentosum* (Ericaceae)

R. tomentosum is a woody evergreen shrub commonly called weeshichbuksh (Cree) or northern Labrador tea (English). *R. tomentosum* is endemic to the circumpolar Subarctic with a short snow-free growing season and long daylight exposures. It is amongst the most commonly used medicinal plants in multiple Canadian First Nations populations for the treatment of type 2 diabetes symptoms [9–10], respiratory illnesses [60], infection [20] and other uses. There is a strong consensus amongst populations for selected usage of *R. tomentosum* for stomach ache, cold symptoms and toothache. Labrador tea, *R. groenlandicum,* has overlapping habitat in subboreal climates with northern Labrador tea and is often used interchangeably with an occasional preference for using the smaller northern Labrador tea for children. These species differ phytochemically, at least in their volatile oil contents, with *R. groenlandicum* having more germacrone and *R. tomentosum* more ledol [19].

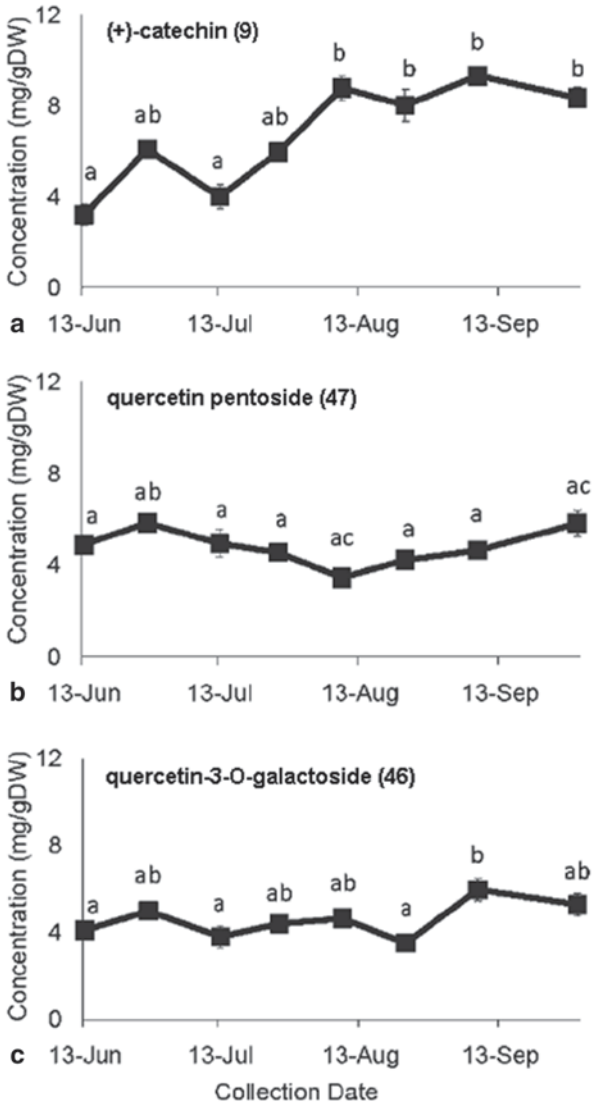


Fig. 4.15 Seasonal variation of the mean quantities ± SEM of the three most abundant compounds in *R. tomentosum* leaf extract; **a** (+)-catechin (9), **b** quercetin pentoside (47), and **c** quercetin 3-O-galactoside (46). Different *letters* indicate significant differences among phytochemical concentrations at various collection dates at $p \leq 0.05$. (Adapted with permission from [57])

Our team identified and quantified a total of 15 phytochemicals in *R. tomentosum* leaf extract throughout their growing season. HPLC-DAD with a detection at 325 nm was used to quantify chlorogenic acid (50), *p*-coumaric acid (39), myricetin (51), quercetin (44), quercetin 3-*O*-galactoside (46), quercetin 3-*O*-glucoside (45), quercetin pentoside (47), quercetin 3-*O*-rhamnoside (54) and three caffeic acid derivatives (unknown chemical structures), whereas detection at 280 nm enabled the identification and quantification of (+)-catechin (9) and three procyanidins

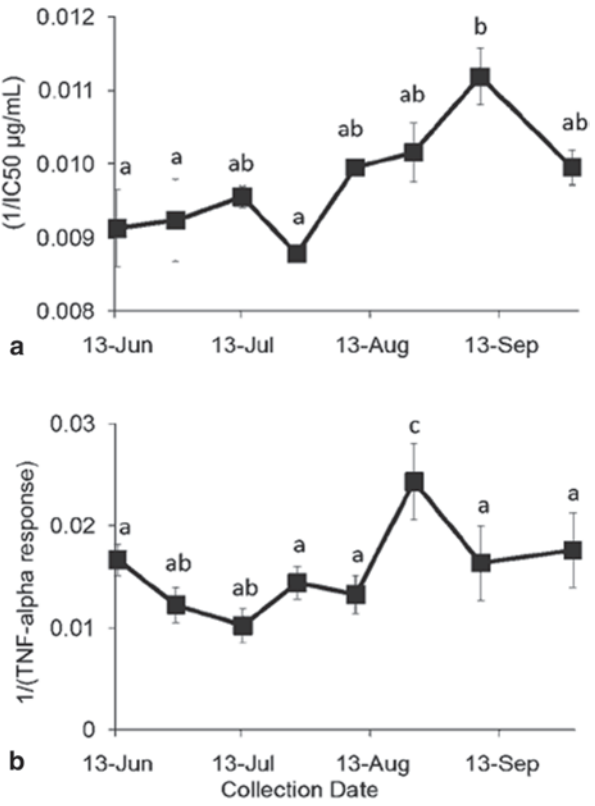


Fig. 4.16 Seasonal variation ± SEM of *R. tomentosum* leaf extract for; **a** antioxidant DPPH activity (1/(IC_{50} µg/mL)) and **b** anti-inflammatory TNF-α activity (1/(% TNF-α relative to vehicle control)). Different *letters* indicate significant collection date differences at $p \leq 0.05$. (Adapted with permission from [57])

(B1 (55), B2 (52) and B3 (56)). The additional chemical structures of the isolated compounds from *R. tomentosum* are shown in Fig. 4.14.

Of the 15 constituents quantified, compounds 9, 47 and 46 were the most abundant, in agreement with previous findings for *R. tomentosum* [59, 61]. These compounds showed a seasonal average of 6.75, 4.79 and 4.58 mg/g DW (milligrams of extract per gram of plant dry weight), and concentration ranges of 3.21–10.2, 3.44–5.83 and 3.51–5.96 mg/gDW, respectively. The quantities of all three compounds were at maxima in September when the leaves of *R. tomentosum* turn red (Fig. 4.15). Similarly, compounds 56 and 52—the fourth and fifth most abundant compounds—displayed seasonal maxima in September. Compound 55 was one of the least abundant compounds with a maximal seasonal average quantity in June. Compounds 50 and 39 had relatively low seasonal average concentrations and displayed similar seasonal trends with a maximal concentration at the end of June. In contrast, the quantities of the three caffeic acid derivatives did not follow any seasonal trend. Compounds 51, 44, 45 and 54 had relatively low seasonal average concentrations. Compounds 50 and 39 had relatively low seasonal average concentrations and displayed similar seasonal trends, with a maximal concentration at

the end of June. The sum of the 15 phenolic compounds did not vary significantly throughout the season; however, there was a clear growth dilution trend [62].

As expected, the DPPH antioxidant activity of *R. tomentosum* displayed a seasonal trend similar to that of the total sum of compounds, whereas there was no such relationship with the TNF-α anti-inflammatory activity (Fig. 4.16). There was a significant correlation between antioxidant activity DPPH IC_{50} and the total sum of phenolic constituents ($r = -0.77$; $p = 0.02$), a trend that has been well established [63, 64]. There was no correlation between anti-inflammatory activity and total sum of constituents. The antioxidant activity found in the *R. tomentosum* leaf extract was generally in agreement with previously reported values [9–10], despite varying sampling locations, seasonal collection dates and testing procedures [61]. Antioxidant activity was not correlated to anti-inflammatory activity, despite the well-characterized use of phenolics as anti-inflammatory agents. Inflammatory signalling cascades include free radicals, which lead to the activation of nuclear factor κB and the transcription of TNF-α inflammatory cytokine [65]. Many traditionally used medicinal plants and phytochemical compounds, such as parthenolide (used as positive control in our study), are known to target these pathways as anti-inflammatory therapies [64].

Considering the widespread use of *R. tomentosum* as a medicinal plant by First Nations and Inuit peoples of Canada, it was important to characterize its phenolic constituents and medicinal activities, as well as to determine how these properties vary over time. The seasonal variation of phenolic constituents and medicinal properties has implications for optimal harvest time and consistency of product. Our results show a significant seasonal variation of phenolic constituents and medicinal properties of *R. tomentosum,* with an optimal harvest time at the end of August to September for our study location, which corroborated traditional knowledge.

4.11 Conclusions and Remarks

While the medicinal phytochemistry of traditionally used plants has been intensively studied in Europe, Asia, Latin America and Africa, plants from temperate North America are lacking such analyses. The formation of the CIHR Team in Aboriginal Antidiabetic Medicines was therefore the first major research project funded by Canada for the study of an important and still actively used traditional medicine system of North America. Clearly, the traditional medicine of the Cree possesses a strong pharmacological [7] and phytochemical basis and can take its place amongst other well-studied world traditional medicine systems. The project also sets a strong example for the mutually respectful collaboration between scientists and Cree Healers. Finally, the research agreement that governs the research programme of the CIHR team in Aboriginal Antidiabetic Medicines sets international precedents in terms of the protection of Cree traditional knowledge and the management of the intellectual property generated by the collaborative studies [66].

Acknowledgments This work was supported by a Team Grant from the Canadian Institutes of Health Research (CIHR Team in Aboriginal Antidiabetic Medicines) to PSH, JTA and AC and was conducted with the consent and support of the Cree communities of Mistissini, Whapmagoostui, Nemaska, Waskaganish, Weminji and Oujé-Bougoumou as well as of the Cree Board of Health and Social Services of James Bay (Quebec, Canada). Very special thanks are due to Elizabeth Coon Come, Mable Gunner, Charlotte Husky Swallow, Johnny Husky Swallow, Ronny Loon and Girty Loon from the Cree Nation of Mistissini, to Eliza Kawapit, Abraham Mamianskum, Andrew Natachequan, Maggie Natachequan and John Petagumskum from Whapmagoostui First Nation, as well as 54 other Elders and Healers who kindly agreed to be interviewed. They made this manuscript possible by allowing us to use, for the purposes of this research, their knowledge relating to medicinal plants, transmitted to them by their Elders. Their trust has also enabled a useful exchange between Indigenous knowledge and Western science. We also wish to dedicate this paper to the memory of the Elders that have passed away in the course of this project.

References

1. Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047–1053
2. Roglic G, Unwin N (2010) Mortality attributable to diabetes: estimates for the year 2010. Diabetes Res Clin Pr 87:15–19
3. Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pr 87:4–14
4. Whiting D, Unwin N, Roglic G (2010) Diabetes: equity and social determinants. In: Blas E, Sivasankara Kurup A (eds) Priority public health conditions: from learning to action on social determinants of health. WHO, Geneva, pp 77–94
5. Kuzmina E, Dannenbaum D, Torrie J (2010) Cree Diabetes Information System (CDIS) 2009 Annual report. Cree Board of Health and Social Services of James Bay. Chisasibi, Québec
6. Leduc C, Coonishish J, Haddad PS, Cuerrier A (2006) Plants used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the treatment of diabetes: a novel approach in quantitative ethnobotany. J Ethnopharmacol 105:55–63
7. Haddad PS, Musallam L, Martineau LC, Harris C, Lavoie L, Arnason JT, Foster B, Bennett S, Johns T, Cuerrier A, Coon Come E, Coon Come R, Diamond J, Etapp L, Etapp C, George J, Husky Swallow C, Husky Swallow J, Jolly M, Kawapit A, Mamianskum E, Petagumskum J, Petawabano S, Petawabano L, Weistche A, Badawi A (2012) Comprehensive evidence-based assessment and prioritization of potential antidiabetic medicinal plants: a case study from Canadian Eastern James Bay Cree traditional medicine. Evid Based Complementary Altern Med. doi:10.1155/2012/893426
8. Spoor DC, Martineau LC, Leduc C, Benhaddou-Andaloussi A, Meddah B, Harris C, Burt A, Fraser MH, Coonishish J, Joly E, Cuerrier A, Bennett SA, Johns T, Prentki M, Arnason JT, Haddad PS (2006) Selected plant species from the Cree pharmacopoeia of northern Quebec possess anti-diabetic potential. Can J Physiol Pharmacol 84:847–858
9. Harbilas D, Martineau LC, Harris CS, Adeyiwola-Spoor DC, Saleem A, Lambert J, Caves D, Johns T, Prentki M, Cuerrier A, Arnason JT, Bennett SA, Haddad PS (2009) Evaluation of the antidiabetic potential of selected medicinal plant extracts from the Canadian boreal forest used to treat symptoms of diabetes: part II. Can J Physiol Pharmacol 87:479–492
10. Fraser MH, Cuerrier A, Haddad PS, Arnason JT, Owen PL, Johns T (2007) Medicinal plants of Cree communities (Québec, Canada): antioxidant activity of plants used to treat type 2 diabetes symptoms. Can J Physiol Pharmacol 85:1200–1214
11. Hosie RC (1979) Native trees of Canada. Fitzhenry & Whiteside, Toronto
12. Leighton AL (1985) Wild plant use by the woods Cree (Nihithawak) of East-Central Saskatchewan. National Museums of Canada, Ottawa

13. Marshall S, Chikamish-Napash L, Lavallée C (2006) The gift of healing, health problems and their treatments. Cree Board of Health and Social Services of James Bay. Chisasibi
14. Guerrero-Analco JA, Martineau L, Saleem A, Madiraju P, Muhammad A, Durst T, Haddad PS, Arnason JT (2010) Bioassay-guided isolation of the antidiabetic principle from Sorbus decora (Rosaceae) used traditionally by the Eeyou Istchee Cree First Nations. J Nat Prod 73:1519–1523
15. Vianna R, Brault A, Martineau LC, Couture R, Arnason JT, Haddad PS (2011) In vivo antidiabetic activity of the ethanolic crude extract of Sorbus decora C.K.Schneid. (Rosaceae): A medicinal plant used by Canadian James Bay Cree Nations to treat symptoms related to diabetes. Evid Based Complement Alternat Med. doi:10.1093/ecam/nep158
16. Holmes EM (1884) Medicinal plants used by the Cree Indians, Hudson's Bay territory. Pharm J Trans 15:302–304
17. Marshall S, Diamond L, Blackned S (1989) Healing ourselves, helping ourselves: the medicinal use of plants and animals by the people of Waskaganish. Cree Regional Authority, Val d'Or
18. Siegfried EV (1994) Plant use by the Wabasca/Desmarais Cree, Ethnobotany of the Northern Cree of Wabasca/Desmarais. National Library of Canada, Ottawa, pp 99–169
19. Marles RJ, Clavelle C, Monteleone L, Tays N, Burns D (2000) Aboriginal plant use in Canada's Northwest boreal forest. University of British Columbia Press, Vancouver, pp 368
20. Arnason JT, Hebda R, Johns T (1981) Use of plants for food and medicine by Native Peoples of Eastern Canada. Can J Botany 59:2189–2325
21. Black MJ (1980) Algonquin Ethnobotany: an interpretation of Aboriginal adaptation in Southwestern Quebec. National Museums of Canada, Ottawa
22. Shang N, Guerrero-Analco JA, Musallam L, Saleem A, Muhammad A, Walshe-Roussel B, Cuerrier A, Arnason JT, Haddad PS (2012) Adipogenic constituents from the bark of Larix laricina du Roi (K. Koch; Pinaceae), an important medicinal plant used traditionally by the Cree of Eeyou Istchee (Quebec, Canada) for the treatment of type 2 diabetes symptoms. J Ethnopharmacol 141:1051–1057
23. Pichette A, Lavoie S, Morin P, Mshvildadze V, Lebrun M, Legault J (2006) New labdane diterpenes from the stem bark of Larix laricina. Chem Pharm Bull 54:1429–1432
24. Suresh-Babu K, Tiwari AK, Srinivas PV, Ali AZ, China-Raju B, Rao JM (2004) Yeast and mammalian alpha-glucosidase inhibitory constituents from Himalayan rhubarb Rheum emodi Wall.ex Meisson. Bioorg Med Chem Lett 14:3841–3845
25. Harbilas D, Vallerand D, Brault A, Saleem A, Arnason JT, Musallam L, Haddad PS (2012) Larix laricina, an Antidiabetic alternative treatment from the Cree of Northern Quebec pharmacopoeia, decreases glycemia and improves insulin sensitivity in vivo. Evid Based Complement Alternat Med 2012:296432. doi:10.1155/2012/296432
26. Schnell D (2003) Carnivorous plants of the United States and Canada, 2nd ed., Timber Press, Oregon
27. Muhammad A, Guerrero-Analco JA, Martineau LC, Musallam L, Madiraju P, Nachar A, Saleem A, Haddad PS, Arnason JT (2012) Antidiabetic compounds from Sarracenia purpurea used traditionally by the Eeyou Istchee Cree First Nation. J Nat Prod 75:1284–1288
28. Eid HM, Martineau LC, Saleem S, Muhammad A, Vallerand D, Benhaddou-Andaloussi A, Nistor L, Afshar A, Arnason JT, Haddad PS (2010) Stimulation of AMP-activated protein kinase and enhancement of basal glucose uptake in muscle cells by quercetin and quercetin glycosides, active principles of the antidiabetic medicinal plant Vaccinium vitis-idaea. Mol Nutr Food Res 54:991–1003
29. Fang XK, Gao J, Zhu DN (2008) Kaempferol and quercetin isolated from Euonymus alatus improve glucose uptake of 3T3-L1 cells without adipogenesis activity. Life Sci 82:615–622
30. Leila Z, Angela R, Poliane F, Maria SRB, Moacir GP, Laura DL, Fa̓ltima R, Silva MB (2008) Insulinomimetic effect of kaempferol 3-neohesperidoside on the rat soleus muscle. J Nat Prod 71:532–535
31. Shinji O, Tadahi A, Toshifumi H, Takayuki S (1984) The structures of four diarylheptanoid glycosides from the female flowers of Alnus serrulatoides. J Chem Soc [Perkin I] 8:1635–1642

32. Lee KK, Bahler BD, Hofmann GA, Mattern MR, Johnson RK, Kingston DG (1998) Isolation and structure elucidation of new PKCa inhibitors from Pinus flexilis. J Nat Prod 61:1407–1409
33. Ruben FG, Richard FH, Jie C, Joseph JK (1998) Two acylated diarylheptanoid glycosides from Red Alder bark. J Nat Prod 61:1292–1294
34. Kuroyanagi M, Shimomae M, Nagashima Y, Muto N, Okuda T, Kawahara N, Nakane T, Sano T (2005) New diarylheptanoids from Alnus japonica and their antioxidant activity. Chem Pharm Bull 53:1519–1523
35. Martineau LC, Muhammad A, Saleem S, Hervé J, Harris CS, Arnason JT, Haddad PS (2010) Anti-adipogenic Activities of Alnus incana and Populus balsamifera Bark Extracts, Part II: Bioassay-guided Identification of Actives Salicortin and Oregonin. Planta Med 76:1519–1524
36. Mattes BR, Clausen TP, Reichardt PB (1987) Volatile constituents of balsam poplar: the phenol glycoside connection. Phytochemistry 26:1361–1366
37. Zazworsky D, Nelson-Bolin J, Gaubeca VB (2005) Handbook of diabetes management. Springer, New York
38. Spoor DA, Martineau LC, Arnason JT, Haddad PS (2007) Mechanisms of anti-diabetic activity of extracts of selected medicinal plant species of the Canadian boreal forest. Diabetes Vasc Dis Res 4:156
39. Martineau LC, Adeyiwola-Spoor DC, Vallerand D, Afshar A, Arnason JT, Haddad PS (2010) Enhancement of muscle cell glucose uptake by medicinal plant species of Canada's native populations is mediated by a common, metformin-like mechanism. J Ethnopharmacol 127:396–406
40. Harris CS, Burt AJ, Saleem A, Le PM, Martineau LC, Haddad PS, Bennett SA, Arnason JT (2007) A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of Vaccinium angustifolium. Phytochem Anal 18:161–169
41. Mcintyre KL, Harris CS, Saleem A, Beaulieu LP, Ta CA, Haddad PS, Arnason JT (2009) Seasonal phytochemical variation of anti-glycation principles in lowbush blueberry (Vaccinium angustifolium). Planta Med 75:286–292
42. Ferrier J, Djeffal S, Morgan HP, Vander Kloet S, Redzic S, Cuerrier A, Balick MJ, Arnason JT (2012) Anti-glycation activity of Vaccinium spp. (Ericaceae) from the Sam Vander Kloet Collection for the treatment of type II diabetes. Botany 90:401–406
43. Misra P (2008) AMP activated protein kinase: a next generation target for total metabolic control. Expert Opin Ther Targets 12:91–100
44. Viollet B, Lantier L, Devin-Leclerc J, Hebrard S, Amouyal C, Mounier R, Foretz M, Andreelli F (2009) Targeting the AMPK pathway for the treatment of Type 2 diabetes. Front Biosci 14:3380–3400
45. Winder WW, Thomson DM (2007) Cellular energy sensing and signaling by AMP-activated protein kinase. Cell Biochem Biophys 47:332–347
46. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE (2001) Role of AMP activated protein kinase in mechanism of metformin action. J Clin Invest 108:1167–1174
47. Viollet B, Foretz M, Guigas B, Horman S, Dentin R, Bertrand L, Hue L, Andreelli F (2006) Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. J Physiol 574:41–53
48. Thong FS, Bilan PJ, Klip A (2007) The Rab GTPase-activating protein AS160 integrates Akt, protein kinase C, and AMP activated protein kinase signals regulating GLUT4 traffic. Diabetes 56:414–423
49. Cartee GD, Wojtaszewski JF (2007) Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. Appl Physiol Nutr Metab 32:557–566
50. Winder WW (2001) Energy-sensing and signaling by AMPactivated protein kinase in skeletal muscle. J Appl Physiol 91:1017–1028
51. Reznick RM, Shulman GI (2006) The role of AMP-activated protein kinase in mitochondrial biogenesis. J Physiol 574:33–39
52. Mcgee SL, Hargreaves M (2008) AMPK and transcriptional regulation. Front Biosci 13:3022–3033
53. Bischoff SC (2008) Quercetin: potentials in the prevention and therapy of disease. Curr Opin Clin Nutr Metab Care 11:733–740

54. Eid HM, Ouchfoun M, Brault A, Vallerand D, Mussalam L, Arnason JT, Haddad PS (2011) W9, a medicinal plant from the pharmacopeia of the Eastern James Bay Cree, exhibits anti-diabetic activities in two mouse model of diabetes. Planta Med 77-PF7. doiI:10.1055/s-0031–1282395
55. Kaushik D, O'Fallon K, Priscilla M, Clarkson PM, Dunne CP, Conca KR, Michniak-Kohn B (2012) Comparison of Quercetin Pharmacokinetics following oral supplementation in humans. J Food Sci 77:H231–H238
56. Chartier Ch, Staub H, Goetz P (2005) Monographie médicalisée: Thé du Labrador (lédon du Groenland) (Ledum groenlandicum Oeder). Phytothérapie 2:84–87
57. Zieba RA (1992) Healing and healers among the northern Cree. National Library of Canada, Ottawa
58. Beardsley G (1941) Notes on Cree medicines, based on collections made by I. Cowie in 1892. Pap Mich Acad Sci Arts Lett 28:492
59. Saleem A, Harris CS, Asim M, Cuerrier A, Martineau L, Haddad PS, Arnason JT (2010) A RP-HPLC-DAD-APCI/MSD method for the characterisation of medicinal Ericaceae used by the Eeyou Istchee Cree First Nations. Phytochem Anal 21:328–339
60. Black PL, Arnason JT, Cuerrier A (2008) Medicinal plants used by the Inuit of Qikiqtaaluk (Baffin Island, Nunavut). Botany 86:157–163
61. Black P, Saleem A, Dunford A, Guerrero-Analco J, Walshe-Roussel B, Haddad P, Cuerrier A, Arnason JT (2011) Seasonal variation of phenolic constituents and medicinal activities of Northern Labrador tea, Rhododendron tomentosum ssp. subarcticum, an Inuit and Cree First Nations traditional medicine. Planta Med 77:1655–1662
62. Surveswaran S, Cai YZ, Xing J, Corke H, Sun M (2010) Antioxidant properties and principal phenolic phytochemicals of Indian medicinal plants from Asclepiadoideae and Periplocoideae. Nat Prod Res 24:206–221
63. Mustafa RA, Abdul-Hamid A, Mohamed S, Bakar FA (2010) Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. J Food Sci 75:C28–C35
64. Winrow VR, Winyard PG, Morris CJ, Blake DR (1993) Free radicals in inflammation: second messengers and mediators of tissue destruction. Br Med Bull 49:506–522
65. D'acquisto F, May MJ, Ghosh S (2002) Inhibition of nuclear factor kappa B (NFkB): an emerging theme in anti-inflammatory therapies. Mol Interv 2:22–35
66. Cuerrier A, Downing A, Patterson E, Haddad PS (2012) Aboriginal antidiabetic plant project with the James Bay Cree of Québec: an insightful collaboration. J Enterp Comm People Places Global Econ 6:251–270

Chapter 5
Bioproducts, Biofuels, and Perfumes: Conifer Terpene Synthases and their Potential for Metabolic Engineering

Philipp Zerbe and Jörg Bohlmann

Abstract Conifer trees, including the economically and ecologically important pine (*Pinus*), spruce (*Picea*), and fir (*Abies*) species, produce large amounts of oleoresin terpenoids as a defense against herbivores and pathogens. Due to the structural diversity of oleoresin terpenoids and their various chemical and physical properties, which range from solid and viscous resins to liquids and volatiles, many of these compounds are useful to humans for the production of therapeutics, fragrances and flavors, biofuels, and fine chemicals. In this chapter, we feature three examples of conifer terpenoids, the diterpene resin acids (DRA), the sesquiterpene *E*-α-bisabolene, and the diterpenol *cis*-abienol, to highlight the versatile utility of conifer terpenoids as renewable bioproducts. We focus on recent research progress on conifer terpene synthases (TPS) which produce a wealth of terpene scaffolds in nature. Our recent advances in conifer transcriptome and genome sequencing as well as metabolite analyses have accelerated discovery and definitive functional annotation of terpenoid pathway genes. New insights into the evolutionary diversification of conifer TPS, their modular organization, and dynamic expression will be fundamental to advance metabolic engineering and synthetic biology platforms for high-value terpenoids.

Keywords Conifer defense · Plant specialized metabolism · Terpene synthase · Metabolic engineering · Bioproducts · Biofuel

5.1 Introduction: Conifer Terpenoids

Terpenoids, or isoprenoids, are a large group of tens of thousands of linear or cyclic natural products, the biosynthesis of which originates from common five-carbon (C_5) building blocks [1, 2]. The many known functions of terpenoids in plant development, general (i.e., primary) metabolism, and in the chemical ecology of

J. Bohlmann (✉) · P. Zerbe
Michael Smith Laboratories, University of British Columbia, 301–2185 East Mall, Vancouver, BC V6T 1Z4, Canada
e-mail: bohlmann@mail.ubc.ca

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application*,
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_5

plants interacting with their environment reflect the extraordinary chemical diversity of these metabolites [3, 4]. The majority of plant terpenoids represent specialized (i.e., secondary) metabolites, some of which are of narrow taxonomic distribution (e.g., taxol) while others appear to be present in many different plant species (e.g., simple monoterpenes such as myrcene, limonene, or pinenes). Some of these specialized compounds may facilitate survival of the producing plant species in hostile environments. For example, coniferous trees of the pine family (Pinaceae) are among the tallest and the longest living organisms on earth, exposed to many potential herbivores and pathogens. Production, accumulation, and secretion of copious amounts of terpenoids in the form of oleoresin provides, in general and under healthy plant and environmental conditions, a durable defense system against pests and pathogens [5–10]. Mono- and diterpenes, including diterpene resin acids (DRA), constitute the most abundant components of conifer oleoresin, with smaller amounts of sesquiterpenes [6, 11–14]. The viscous and hydrophobic oleoresin accumulates in resin cells, resin blisters, or resin ducts in different tissues and organs of conifer trees. Formation of these compounds in specialized secretory epithelial cells of resin ducts is constitutive and inducible [15–18]. Upon wounding, the resin is released from storage reservoirs and moves under pressure to the tree surface or into wound sites within tissues, where the resin can physically and chemically repel or kill herbivores and pathogens. After evaporation of the volatile mono- and sesquiterpenes, the remaining oleoresin compounds, mostly DRA, polymerize and seal off the wound site [7, 11].

The complex chemical and physical defense system of conifer oleoresin has proven effective against many mammalian herbivores, insect pests, and microorganisms, and has allowed conifer trees to evolve and thrive as the dominant group of gymnosperm plants on earth. However, as with any plant defense system, certain specialist herbivores and pathogens have evolved with the oleoresin defenses of conifers and have developed behavioral and biochemical strategies to cope with these chemicals [9]. For example, mass attack of bark beetles, such as the mountain pine beetle (*Dendroctonus ponderosae*), and their associated microbial communities (e.g., ophiostomatoid blue staining fungi) have destroyed more than 15 million ha of pine forest across western North America in the past 10–20 years, with dramatic impacts on the ecosystems of entire landscapes and on the economies of forestry-dependent industries and communities [8, 19, 20]. Applied genomics and systems research of the mountain pine beetle system (e.g., the Tria Project) has revealed some of the molecular and genetic intricacies and forces of the interacting pine, bark beetle, and microbial components of the mountain pine beetle system, including chemical interactions mediated by pine terpenoid defenses and adaptations to these defenses by specialist insects and associated microorganisms [8, 21–26]. For example, behavioral and molecular adaptations of the mountain pine beetle include mass attack of pine hosts, which involves detoxification and metabolism of host monoterpenes to produce insect pheromones. Molecular adaptations of the mountain pine beetle-associated fungal pathogen *Grosmannia clavigera* to its pine host include transport (export) and metabolism of monoterpenes and utilization of some monoterpenes as a carbon source to support fungal growth [24].

Paralleling the diversity and importance of hundreds of different terpenoid compounds in conifer defense, practical uses of conifer terpenoids by humans are also manifold [12]. For centuries, conifer terpenoids in the form of plant extracts, resins, or essential oils have been used as traditional medicines, fragrances, or as incense for religious and other ceremonial purposes. As a large-volume raw material, conifer terpenoids have long been used in the traditional naval stores industries. In present times, owing to their unique and often pleasant odor characteristics, volatile mono- and sesquiterpenoids of conifers serve in large-volume feedstocks for fragrance and food industries with an annual production volume of several hundred thousand metric tons. Limonene and pinenes produced in conifers and in other plant species are some of the major commercially used terpenoids, commonly found as ingredients in numerous household and industrial cleaning products, paint thinners and solvents, cosmetic and hygiene products, air fresheners, and a multitude of other consumer articles of everyday modern life [27] (Fig. 5.1). Some terpenoids, specifically β-pinene, farnesene, and *E*-α-bisabolene of conifer or other plant origin, are developed as potential biofuel alternatives to conventional petrochemicals [28].

The nonvolatile rosin fraction of oleoresin is also commercially important, with more than one million metric tons produced yearly for applications, ranging from adhesives and solvents over industrial coatings to printing inks [29, 30]. As the major rosin component, DRA attract increasing interest in the synthesis of designer polymers for long-term replacement of petroleum-based plastics [30, 31]. The antimicrobial properties of conifer DRA are further being considered for medicinal applications [32]. While a mixture of DRA are present in many conifer species of the pine family, some conifer terpenoids are more narrowly restricted in their taxonomic distribution. For example, the diterpene alcohol *cis*-abienol is abundant in the bark tissue of balsam fir (*Abies balsamea* (L.) Mill.) and can be used as a direct precursor for synthesizing ambroxides (Ambrox® is trademarked by Firmenich, Geneva), which are fixatives in some high-value perfume formulations [33]. Another prominent example of a conifer diterpenoid of limited natural occurrence is paclitaxel (Taxol® is a trademark of Bristol-Myers Squibb, New York), the most widely employed anticancer drug worldwide (Fig. 5.1). Paclitaxel can be found exclusively in species of yew (*Taxus* spp.) and was traditionally harvested from the trees' bark tissue [34]. However, the increasing demand for cancer chemotherapy made this slow-growing tree a limited and dwindling natural resource [35]. In the absence of economically feasible total synthesis procedures, extensive efforts have been undertaken to identify the enzymes involved in the biosynthesis of taxol *in planta* and their complex catalytic mechanisms [36], which can be applied to metabolic engineering of taxol (comprehensively reviewed in [36, 37]). Today, taxol is produced in large-scale microbial and plant cell culture platforms with an annual market value of several billion US dollars [38, 39]. Other emerging diterpenoid therapeutics of conifer origin include pseudolaric acids found in the roots of golden larch (*Pseudolarix amabilis*, J. Nelson) [40], and ferruginol occurring in the bark of Japanese cedar (*Cryptomeria japonica*, (L.f.) D. Don) and in cones of giant redwood (*Sequoia sempervirens*, (Lamb. *ex* D. Don) Endl.), with efficacy against various cancers [41, 42] (Fig. 5.1).

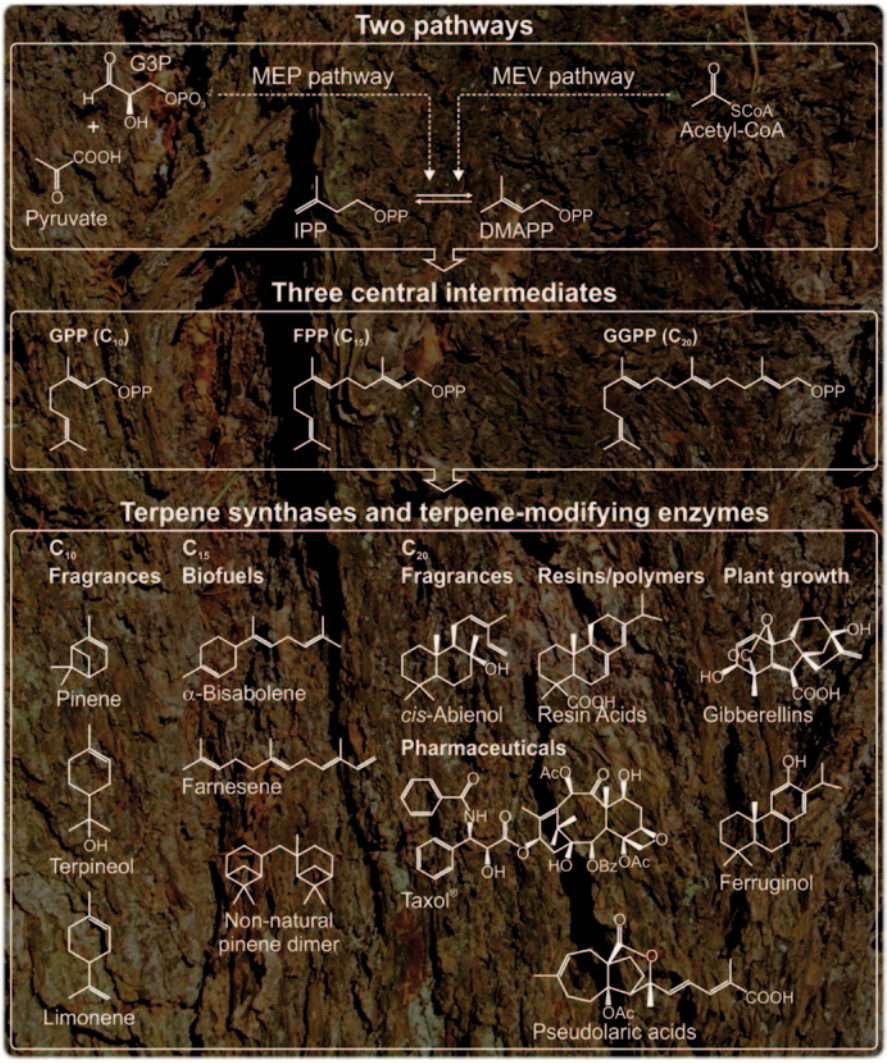


Fig. 5.1 Conifer terpenoid natural products. General overview of the biosynthesis of select conifer-derived natural products. *MEP* 2-*C*-methyl-D-erythritol-4-phosphate, *MEV* mevalonate, *G3P* glyceraldehyde-3-phosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate

Given a continuous interest in new and improved diterpenoid bioproducts for industrial applications, reliance on natural resources alone is often neither sufficient nor sustainable. Chemical synthesis, although successful in several cases [27, 43], has often been impractical and not cost-effective, given the structural and stereochemical complexity of many terpenoids. Following the lead of taxol and artemisinin (an antimalaria sesquiterpene agent derived from *Artemisia annua*, L.)

[44, 45], exciting opportunities exist to develop and enhance the industrial-scale production of high-value terpenoids by metabolic engineering of partial or entire natural biosynthetic pathways in microbial systems, such as *Escherichia coli* and yeast (*Saccharomyces cerevisiae*), as well as in heterologous plant hosts. Key to successful terpenoid pathway engineering is a detailed understanding of the biosynthetic enzymes responsible for the formation of both simple and complex molecules. Advanced methodologies in genomics, proteomics, and metabolomics have accelerated discovery and characterization of relevant genes, enzymes, and metabolites, and are revealing matrix-type metabolic networks of terpenoid biosyntheses in conifers that provide a versatile toolbox for enzymatic small molecule manufacture [33, 46, 47].

In this article, we highlight recent advances in research on the family of conifer terpene synthases (TPS), their functional diversification and possible industrial application. On the basis of three examples, DRA biosynthesis, formation of *cis*-abienol, and *E*-α-bisabolene production, we discuss the economic value of conifer terpenoids and new opportunities to utilize TPS for developing metabolic engineering and synthetic biological approaches for the sustainable production of high-value terpenoid bioproducts.

5.2 TPS-d Enzymes for Terpenoid Biosynthesis in Conifers

Much of today's understanding of enzymes of terpenoid biosynthesis in conifers builds on the fundamental research by Professors Rodney Croteau, Robert Coates, and their coworkers during the last three decades [48, 49]. On this foundation, several gymnosperms, including species of yew (*Taxus*) and spruce (*Picea*) have been established among some of the best studied systems for terpenoid biosynthesis.

Despite their tremendous chemical diversity, all plant terpenoids share a biosynthetic origin in form of two common C_5 building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Fig. 5.1). Two routes exist in plants from which IPP and DMAPP derive, the cytosolic mevalonate (MVA) pathway originating from acetyl-CoA and the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway that derives from pyruvate and glyceraldehyde-3-phosphate [50]. With the exception of hemiterpenes (C_5), formed directly from DMAPP [51], condensation of two, three, or four C_5 units by prenyl transferases affords a few distinct prenyl diphosphate intermediates as central precursors for all terpenoids [52] (Fig. 5.1). Monoterpenes (C_{10}) are derived from geranyl diphosphate (GPP), sesquiterpenes (C_{15}) and triterpenes (C_{30}) are derived from farnesyl diphosphate (FPP), and diterpenes (C_{20}) and tetraterpenes (C_{40}) are formed from geranylgeranyl diphosphate (GGPP).

Immediately downstream of the formation of GPP, FPP, and GGPP, it is the catalytic plasticity (i.e., the ability of enzymes to evolve functional changes with only a few amino acid substitutions) of TPS that drives the initial structural

diversification of terpenoids [2, 52]. TPS catalyze the conversion of prenyl diphosphate substrates into various hydrocarbon or oxygenated terpene scaffolds via multistep, carbocation-driven (cyclo)isomerization reactions [53]. The functional diversity of TPS is rooted in their modular structure of a conserved α-helical folding pattern with variations in three common domains γ, β, and α (for review see [53, 54]). Depending on the presence and composition of either one or two active sites and their associated catalytic motifs, TPS are classified as class I, class II, or class I/II enzymes [2]. Class II enzymes contain a conserved D*x*DD motif and are specific to diterpene metabolism, catalyzing the protonation-initiated cyclization of GGPP into stable bicyclic diphosphate intermediates with differences in stereochemistry and, in some cases, functional modification through capture of water [33, 55, 56]. Other diterpene synthases (diTPS), and likewise all plant monoterpene synthases (monoTPS), and sesquiterpene synthases (sesquiTPS), are class I TPS carrying DD*xx*D and (N/D)x_2(S/T/G)x_3E signature motifs and transforming linear or cyclic (diterpene biosynthesis) diphosphate substrates through cleavage of the diphosphate leaving group and subsequent rearrangement of resulting carbocations. Bifunctional class I/II diTPS that harbor both functional active sites occur in the diTPS of mosses, such as *Physcomitrella patens* ((Hedw.) Bruch & Schimp) and *Jungermannia subulata* (Evans), the lycophyte *Selaginella moellendorffii* [57, 58], and gymnosperms [59]. Cytochrome P450 monooxygenases (P450) and in some instances various transferase enzymes increase the structural diversity of terpenoids in conifers by introducing additional functionalities to the TPS products [46, 60–63].

Current knowledge suggests that only *S. moellendorffii* [64] and conifers contain members of all three TPS classes (I, II, and I/II). An assembly of the 20 Gbp white spruce genome sequence was recently completed [65], which can be used in future work for the genome-wide annotation of TPS genes in a conifer. In the meantime, transcriptome analyses estimated that a single conifer species may contain more than 70 distinct TPS [2, 66], similar to the number of TPS in some well-annotated plant genomes (e.g., 40 in *Arabidopsis* (L.) Heynh. [67], 66 in *S. moellendorffii* [64], and 152 in grapevine (*Vitis vinifera*, L.) [68]). The evolution of these TPS families apparently involved repeated gene duplication events followed by sub- and neofunctionalizations [2]. Conifer TPS of specialized metabolism form the TPS-d family which comprises the monofunctional class I sesquiTPS (TPS-d2) and monoTPS (TPS-d1) and the bifunctional class I/II diTPS as well as monofunctional class I diTPS and diTPS-like enzymes of the TPS-d3 family [2, 47] (Fig. 5.2). We also recently identified the monofunctional class II and class I diTPS *ent*-copalyl-diphosphate synthase and *ent*-kaurene synthase, respectively, in spruce [56]. These latter enzymes group with angiosperm enzymes of the TPS-c and TPS-e/f families of general metabolism, suggesting that diTPS of gibberellin biosynthesis are conserved across angiosperms and gymnosperms.

While the evolutionary diversification of TPS functionalities in the angiosperms is thought to have arisen from diTPS of gibberellin biosynthesis [55], the diversity of conifer TPS functions appears to have evolved from bifunctional diTPS ancestors, which may have been similar in structure and function to *P. patens ent*-kaurene synthase [2, 57]. This model is underscored by recent breakthroughs in the structure elucida-

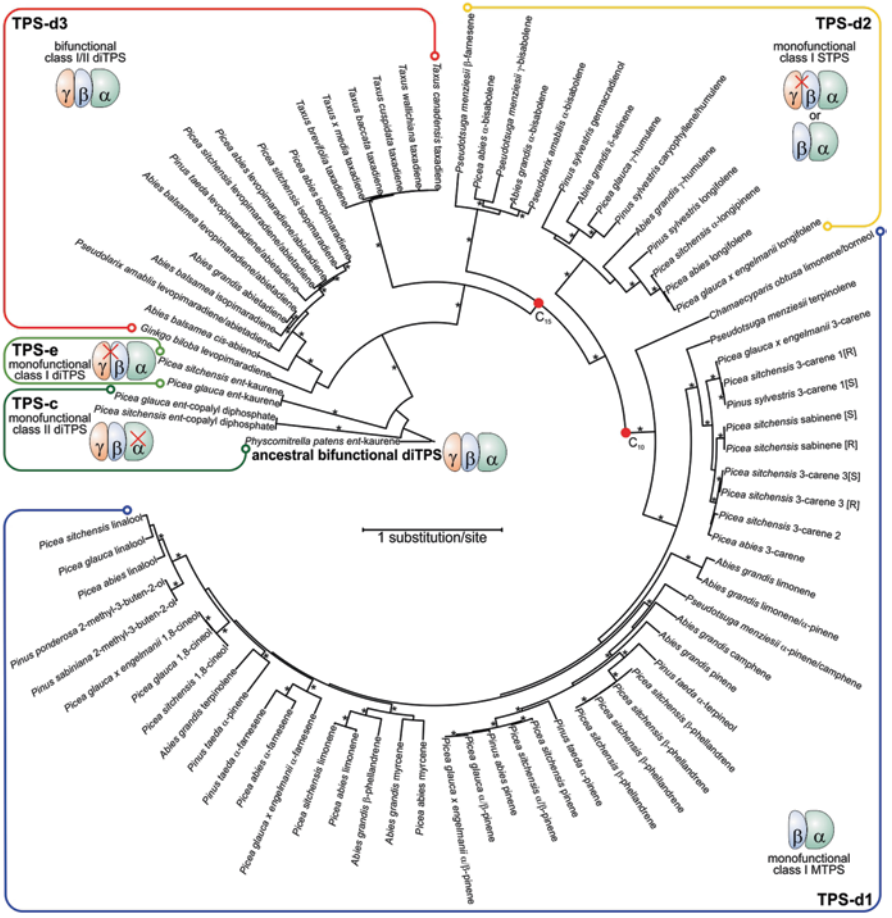


Fig. 5.2 Conifer terpene synthase phylogeny. Maximum likelyhood tree of functionally characterized gymnosperm terpene synthases. *Physcomitrella patens ent*-kaurene/kaurenol synthase was used to root the tree. Branches indicated with *asterisks* represent bootstrap support greater than 80% (100 repetitions). Alterations in the substrate specificity from GGPP (C_{20}) to FPP (C_{15}) and GPP (C_{10}) are *highlighted*. Modifications in the typical γβα-domain architecture of TPS and the presence of functional active sites (a *red cross* indicates loss of function) are illustrated corresponding to the individual subfamilies

tion of several diTPS and diTPS-like enzymes, which revealed a conserved domain architecture among all TPS classes [54, 69–71]. Based on the conserved TPS-folding pattern, Cao et al. developed a unifying model of TPS evolution, proposing that a prototypical γβα-domain architecture originated from ancient gene fusion events between bacterial γβ- and α-domain diTPS, which themselves evolved from archetypical γβ-domain triterpene synthase and α-domain prenyl transferase-type progenitors [72]. The large group of βα-domain monoTPS and sesquiTPS from the TPS-d2 and TPS-d1 families are the result of subsequent events of monofunctionalization and loss of the

γ-domain and/or the plastidial transit peptide [2] (Fig. 5.2). The recently solved crystal structure of grand fir (*Abies grandis*, (Douglas *ex* D. Don) Lindl.) *E*-α-bisabolene synthase (*Ag*BIS) is in agreement with this evolutionary path, as it adopts the ancestral γβα-domain architecture similar to diTPS members of the TPS-d3 family, but carries a vestigial class II active site and a substantially modified class I active site toward FPP rather than GGPP conversion [73].

Bifunctional class I/II diTPS appear to have been retained only in non-seed plants [57, 58] and gymnosperms [59]. In the gymnosperms, the bifunctional class I/II diTPS constitute the TPS-d3 family, which also includes γβα-domain sesquiTPS, such as *Ag*BIS, and the taxadiene synthases of the yew genus (*Taxus*) [2] (Fig. 5.2). Bifunctional conifer diTPS include the levopimaradiene/abietadiene synthases (LAS) and isopimaradiene synthases (ISO) of DRA metabolism [47, 74, 75], as well as the bifunctional *cis*-abienol synthase of balsam fir (*Ab*CAS) [33]. Interestingly, *Ab*CAS is phylogenetically equidistant between the LAS from *Ginkgo biloba* (L.) and LAS and ISO enzymes of other conifer species, suggesting that this function evolved prior to the speciation of spruce and fir and was lost in other conifer species (Fig. 5.2) [33]. The recent discovery of *Ab*CAS highlights the power of genomic and transcriptomic approaches to elucidate novel TPS functionalities.

5.3 Biosynthesis of Conifer Diterpene Resin Acids and Applications

In the last 5–10 years, much has been learned about the diTPS and P450 of DRA biosynthesis in conifers. Bifunctional class I/II conifer diTPS form various abietane and pimarane scaffolds via conversion of GGPP into (+)-copalyl diphosphate (CPP; i.e., CPP of normal stereochemistry) and subsequent rearrangement of distinct secondary and tertiary carbocations. DiTPS of the LAS type were known for the last decade to produce multiple abietane products, while ISO-type synthases appear to be single-product enzymes [33, 47, 74–76]. A recent detailed functional analysis of Norway spruce LAS identified a novel, previously overlooked, epimeric diterpene alcohol 13-hydroxy-(8, 14)-abietene (Fig. 5.3) as the initial product of this enzyme [77]. The 13-hydroxy-(8, 14)-abietene product is formed during the class I reaction of LAS through hydroxylation of an abietadienyl carbocation [77]. These tertiary alcohol epimers are unstable and in vitro (and perhaps also in vivo) dehydrate into the previously reported LAS products, abietadiene, palustradiene, levopimaradiene, and neoabietadiene. Formation of the diterpenol 13-hydroxy-(8, 14)-abietene as a product was confirmed for LAS enzymes of balsam fir [33], jack pine (*Pinus banksiana*, Lamb.), and lodgepole pine (*Pinus contorta*, Douglas *ex* Loudon) [47], as well as golden larch (*P. amabilis*; Zerbe et al., unpublished) and appears to be specific to LAS, whereas isopimaradiene formation proceeds directly to the diterpene olefin product. While the enzyme structural features that control the formation of 13-hydroxy-(8, 14)-abietene are unresolved, investigation of the crystal structure of grand fir LAS [71] may shed new light on the single versus multiproduct nature of LAS-type diTPS.

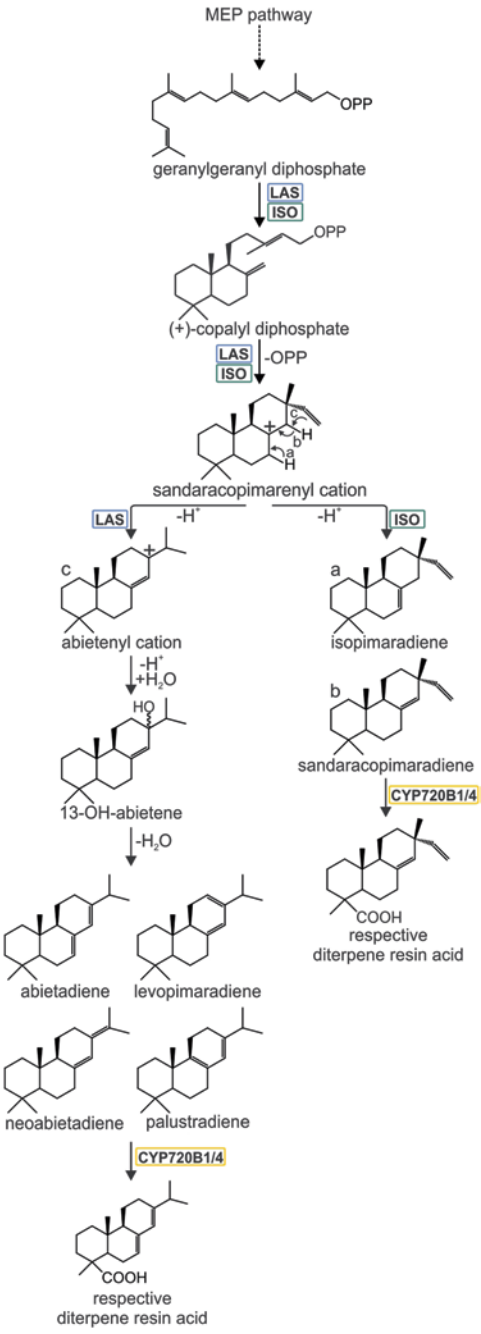


Fig. 5.3 Diterpene resin acid biosynthesis. Diterpene resin acids are formed through the activity of bifunctional class I/II diTPS that convert GGPP into tricyclic labdane-diterpene scaffolds via distinct carbocation intermediates. Carboxylation at position C_{18} is then mediated by cytochrome P450-monooxygenases to afford the respective diterpene resin acids

The olefin products of LAS and ISO enzymes are substrates for the P450-dependent formation of the corresponding C_{18}-alcohols, aldehydes, and DRA. The P450s of DRA biosynthesis are members of the conifer-specific CYP720B family [46, 60]. Genome and transcriptome mining of several spruce species showed that CYP720B enzymes belong to a small gene family of approximately 12 members in a single species [46, 60]. Transcripts of Sitka spruce CYP720B4 were strongly inducible by methyl jasmonate and abundant in resin ducts as the primary location of DRA biosynthesis [46]. In vitro and in vivo functional analysis demonstrated CYP720B4 as a multifunctional and multi-substrate enzyme that facilitates the three-step oxidation of all major conifer diterpene olefins to the respective C_{18}-alcohols, aldehydes and ultimately DRA. Future studies are needed to elucidate the functions of other members of the CYP720B family. Preliminary work suggests that some members of the CYP720B family can convert the olefin products of LAS and ISO, while others can use the 13-hydroxy-(8, 14)-abietene diterpenol as a substrate (unpublished work from our laboratory).

The DRA formed by conifer diTPS and P450s are released upon insect attack and polymerize when exposed to oxygen and UV light into a solid material [7]. While the DRA polymers act as a wound sealant in nature, it can also be used for the production of industrial polymeric materials, including resins, rubbers, and plastics [31, 32]. Even though the mechanistic details of the polymerization are not yet fully understood, chemical synthesis of tailored polymers based on tree-derived resin feedstock is possible [32]. With increasing numbers of characterized diTPS and diterpene-modifying P450s, microbial production platforms attract attention as an alternative source for diterpene-based polymers. Combinatorial expression of diTPS and P450s in yeast for laboratory-scale biosynthesis of abietane- and pimarane-type diterpene monomers has been established [46]. This provides a promising tool for future synthetic biology approaches to produce designer polymers with tailored stereo-specificity and intermolecular linkage that may possess enhanced properties and biodegradability [31].

5.4 Biosynthesis of *cis*-abienol, a Perfume Precursor from Trees

cis-abienol is a relatively rare labdane-type diterpene alcohol found in a few species of the Solanaceae, Asteraceae, and Pinaceae [78–81]. Balsam fir (*Abies balsamea*) produces large amounts of *cis*-abienol that can account for up to 40 % of the aromatic oleoresin accumulating in resin ducts and blisters [33]. Historically, balsam fir oleoresin was known as “Canada Balsam” for its use as an antifouling component in naval stores [78, 82]. Today, diterpene alcohols, such as *cis*-abienol, sclareol, and manool are important in the fragrance industry for the production of ambroxide compounds which substitute for ambergris (grey amber). Ambergris and ambroxides are valued in high-end perfume compositions for their musky and sweet earthy notes and fixative properties [83, 84]. Natural ambergris was traditionally collected

as a scarce biliary secretion from sperm whales, an endangered species. Ambrox® is synthesized from the labdanoid diterpenediol sclareol, which is harvested from cultivated clary sage (*Salvia sclarea*, L.) [85]. The recent discoveries of the diTPS genes and enzymes for the biosynthetic pathways for *cis*-abienol [33] and sclareol [84, 86] created new opportunities for the biotechnological production of ambergris substitutes for perfume manufacture.

For the discovery of *cis*-abienol synthase (CAS) [33], we used high-throughput transcriptome sequencing of balsam fir bark tissue. Assembled transcriptome sequences were searched for diTPS genes using expert annotated TPS protein sequence databases developed in our program. This approach identified three new bifunctional class I/II diTPS. Enzyme characterization showed that *Ab*LAS and *Ab*ISO were paralogous to other conifer LAS and ISO enzymes involved in DRA biosynthesis, respectively, and a third more divergent candidate was a novel diTPS producing *cis*-abienol as a single product [33]. *Ab*CAS transforms GGPP in a cyclohydration reaction to labda-13-en-8-ol diphosphate (LPP) in the enzyme's class II active site, followed by cleavage of the diphosphate ester without further cyclization in the class I active site (Fig. 5.4). Elucidation of the catalytic specificity of *Ab*CAS confirmed that diTPS can form hydroxylated products without requirement for P450-mediated oxygenation. Formation of diterpenol products by diTPS has also been described for the non-seed plants *P. patens* and *S. moellendorffii* [57, 58], several angiosperms [81, 84, 87], and Norway spruce LAS [76]. The functional divergence of *Ab*CAS relative to LAS and ISO enzymes is reflected by its distinct position in the TPS-d3 phylogeny (Fig. 5.2). Several active site residues are unique in the *Ab*CAS class II and class I active sites when compared with other bifunctional conifer diTPS. The underlying changes may have been critical in a neofunctionalization for producing a bicyclic diterpene alcohol instead of tricyclic diterpene olefins. Indeed, substitution of one of the respective residues in the bifunctional abietadiene synthase from grand fir (*A. grandis*; *Ag*AS) led to an alteration of the class II activity from producing (+)-CPP to LPP [88]. Future structural and functional analysis of *Ab*CAS can provide particular insight into the mechanism and plasticity of the class II reaction which is not well understood.

In contrast to the formation of *cis*-abienol by a single bifunctional class I/II diTPS protein in balsam fir, formation of the same metabolite in tobacco is catalyzed by a pair of sequentially acting monofunctional class II and class I diTPS of the TPS-c and TPS-e/f families, with the class II enzyme transforming GGPP into LPP, and the class I diTPS facilitating the subsequent formation of *cis*-abienol [81]. Apparently, the diTPS genes for *cis*-abienol biosynthesis evolved independently in gymnosperms and angiosperms, illustrating a case of convergent functional specialization. The active site residues found to be unique in *Ab*CAS, relative to other conifer diTPS of the TPS-d3 family, are not conserved in functionally similar angiosperm diTPS, leaving the mechanistic underpinnings of the cyclohydration reaction yet to be explored. In tobacco, labdanoid diterpene alcohols were recently correlated with pathogen resistance [89].

Ambrox® can be produced from *cis*-abienol in high yield through a three-step chemical synthesis [83] (Fig. 5.4). Natural sources of a *cis*-abienol, specifically balsam fir oleoresin and extracts of tobacco, provide a complex mixture of different

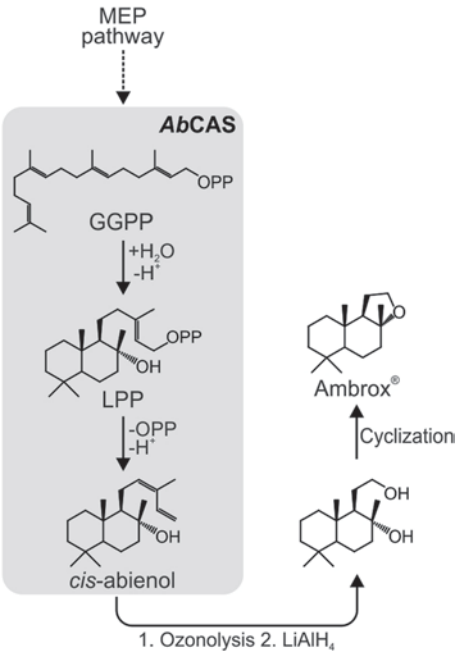


Fig. 5.4 Pathway of *cis*-abienol in balsam fir and chemical synthesis of Ambrox. The biosynthesis of *cis*-abienol in balsam fir (*Abies balsamea*) is catalyzed by a bifunctional class I/II diTPS and proceeds via the initial cyclization of geranylgeranyl diphosphate (GGPP) into a hydroxylated diphosphate intermediate (labda-13-en-8-ol diphosphate, LPP) and subsequent conversion into *cis*-abienol without further cyclization to occur. A two-step chemical conversion of *cis*-abienol affords Ambrox®

diterpenes requiring additional separation and purification. In contrast, microbial systems transformed with a plant diTPS can afford the production of a single defined diterpene target compound. Proof of concept for microbial metabolic engineering of diterpene production has been provided, for example, by overexpression of two monofunctional diTPS from clary sage for production of sclareol in yeast and *E. coli* [84, 86], and by production of taxadiene in *E. coli* using taxadiene synthase [44]. For the microbial engineering or synthetic biology of *cis*-abienol production, *Ab*CAS as a bifunctional enzyme, directly converting GGPP into *cis*-abienol, may have advantages over the use of combinations of monofunctional diTPS from tobacco. Use of *AbCAS* requires expression and optimization of only one gene and provides a naturally evolved scaffolded arrangement for the two critical active sites allowing for efficient channeling of metabolic intermediates.

5.5 Biosynthesis of E-α-bisabolene, a Potential Biofuel Precursor

Conifer terpenoids are being explored as alternative fuel sources to conventional petrochemicals with potentially superior qualities compared to ethanol and biodiesel (for review see [28]). For example, the fully reduced forms of some monoterpenes (e.g. myrcene and limonene) and sesquiterpenes (e.g. farnesene and bisabolene) (Fig. 5.1), show comparable properties to traditional diesel fuel and are being developed as tailored D2 diesel substitutes [90–92]. The cyclic monoterpene pinene

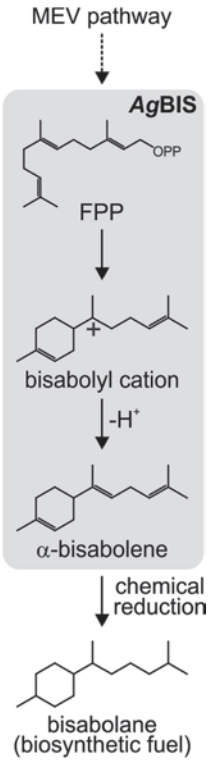


Fig. 5.5 *E*-α-Bisabolene biosynthetic pathway and chemical synthesis of bisabolane. *E*-α-Bisabolene is formed through direct cyclization from farnesyl diphosphate (FPP). Further chemical hydrogenation affords bisabolane as precursor for D2 diesel fuels

can be converted into constrained dimeric ring structures of higher energy content for usage as jet and rocket fuel additives [28, 93]. Large-volume feedstock for these terpenoids is derived from tree and fruit oils. Beyond these natural resources, metabolic engineering and synthetic biology using microbial hosts are being explored, or are already emerging, as alternative approaches for producing terpenoid-based biofuels [28]. In a recent study, Peralta-Yahya et al. highlighted the utility of conifer TPS for biofuel production [91], using a grand fir *Ag*BIS [94].

In conifers, *E*-α-bisabolene is produced as a sesquiterpene component of the oleoresin and can serve as a precursor of todomatuic acid and related insect juvenile hormone III mimics, which may interfere with insect development and reproduction [94, 95]. *Ag*BIS was the first bisabolene synthase to be characterized [94] (Fig. 5.5). Paralogous enzymes exist in Douglas fir (*Pseudotsuga menziesii*, (Mirb.) Franco) and Norway spruce [75, 96]. When developing microbial production systems for *E*-α-bisabolene as a biofuel component, *Ag*BIS was found to be the most catalytically efficient candidate among several conifer γβα-domain BIS and their βα-domain counterparts from angiosperms [91]. Using *E. coli* and yeast host strains, previously engineered for FPP overproduction [45, 97], codon optimization of *Ag*BIS and several key genes in the MEV pathway was combined with additional promoters to generate successful microbial production of *E*-α-bisabolene [91]. Further metabolic engineering through downregulation of competing pathways (i.e., lipid and ergosterol biosynthesis), as well as improved fermentation processes,

achieved production levels of more than 5 g/L culture [98]. Simple chemical reduction of the produced *E*-α-bisabolene afforded α-bisabolane as a possible diesel additive [91] (Fig. 5.5).

The tertiary structure of *Ag*BIS was recently elucidated [73]. Like the conifer bifunctional diTPS, from which it has evolved, *Ag*BIS adopts the ancestral γβα-domain structure, but lacks class II activity and exhibits a smaller class I cavity that accommodates FPP as a substrate rather than GGPP. Structural analysis of *Ag*BIS in the presence of several inhibitors and substrate analogues revealed that the enzyme's catalytic mechanism proceeds via a unique bisabolyl carbocation, and identified several active site residues that determine its catalytic activity. Since the enzymatic efficiency of a TPS can be a limiting factor for microbial formation of a terpenoid product, information from structure/function analyses of *Ag*BIS may offer new leads for enzyme engineering and improved *E*-α-bisabolene production.

Significant progress has been made with the development of metabolically engineered microbial systems for the production of terpenoid biofuel precursors such as *E*-α-bisabolene, farnesene, or pinene [28, 90, 99]. Scaling processes to industrial production and achieving competitive production costs remain challenges for commercialization of terpenoid biofuels. Regardless of any success or possible demise of terpenoid biofuels, the knowledge and technology development that resulted from this work will be broadly transferrable to metabolic engineering and synthetic biology applications of other terpenoids of high value, including pharmaceuticals and fine chemicals.

5.6 TPS Diversity, a Reservoir for Biochemical Engineering

The conifer TPS for the biosynthesis of DRA, *cis*-abienol, and *E*-α-bisabolene provide a few examples of the catalytic capacity of the TPS family in the formation of thousands of natural products. The modular architecture of TPS enzymes and their molecular evolution through recombination and modifications of the typical γβα-domain structure and the composition of class II or class I active sites is fundamental to the functional plasticity of TPS [54,100]. In future work, it should be possible to explore this functional plasticity for rational design of custom-made TPS for the production of desired natural or nonnatural terpenoids, retaining enzymatic control over the stereo- and regio-selectivity of the reaction. In addition, the rapidly growing genome and transcriptome resources are expanding the functional landscape of known TPS, providing a nearly unlimited reservoir of enzymes for biochemical engineering approaches [101, 102]. Following earlier pioneering work on structures of various class I βα-domain mono- and sesqui-TPS from angiosperms [53], the recent solution of structures of four γβα-domain TPS (*Taxus* taxadiene synthase (class I); *Arabidopsis ent*-CPP synthase (class II); grand fir abietadiene synthase (class I/II) and *E*-α-bisabolene synthase (class I) [69–71, 73]) revealed not only a plausible evolutionary relationship of all TPS, but provided fundamental new insights into

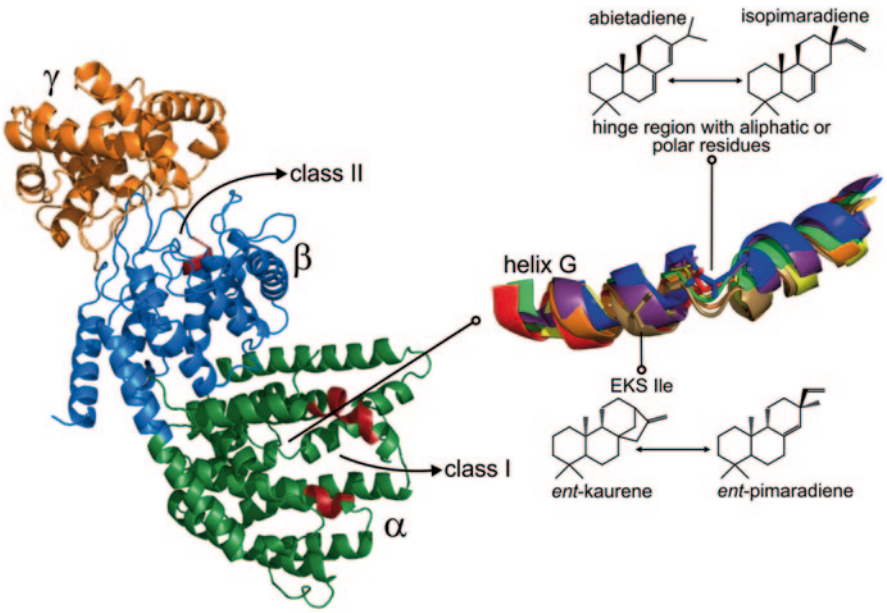


Fig. 5.6 "Hotspots" in the terpene synthase structure are critical for functional divergence. Depicted is the crystal structure of the bifunctional grand fir abietadiene synthase (PDB-ID: 3S9V) that adopts the characteristic three-domain architecture of an N-terminal γ- (*orange*) and β-domain (*blue*) and a C-terminal α-domain (*green*). The catalytic motifs of the class II (D*x*DD) and class I (DDxxD, (N/D)x_2(S/T/G)x_3E) active site are highlighted in *red*. The magnified hinge region on helix G in the α-domain is conserved as shown here by superimposition of representatives of all known TPS classes: grand fir abietadiene synthase (*blue*), Norway spruce isopimaradiene synthase (*red*), white spruce *ent*-kaurene synthase (*brown*), pacific yew taxadiene synthase (*purple*), grand fir α-bisabolene synthase (*orange*), tobacco *epi*-aristolochene synthase (*yellow*), and *Salvia fructicosa* 1, 8-cineol synthase (*green*). Presence of aliphatic or hydrophilic amino acids in this short-loop segment dramatically impact TPS catalytic specificity

the active site contour and composition of some important TPS enzymes (for detailed review see [54]). Certain aspects of TPS mechanisms remain to be explored, such as the conformational transition underlying active site closure, channeling of intermediates between active sites of the same enzyme and different proteins, or the complex steric and electrostatic control that TPS exert during catalysis.

A number of targeted structural/functional analyses demonstrated the ease of functional change in conifer γβα-domain diTPS through as little as a single residue exchange, highlighting select "hotspots" where mutations cause a major change in product specificity [76, 88, 103, 104]. A critical structural feature is the short hinge region on helix G, deeply buried at the bottom of the class I active site cavity (Fig. 5.6). Small changes in the composition of this short loop segment completely alter product outcome. For example, in several bifunctional conifer diTPS, substitution of aliphatic to hydrophilic amino acids and vice versa in this region redirected the catalytic route from abietadiene-type products to pimaradiene-type structures [76, 103]. From these results, it was postulated that introducing a hydroxyl group in

this position will stabilize the intermediate pimarenyl carbocation for deprotonation and formation of pimaradiene, whereas aliphatic residues promote carbocation rearrangement en route to abietadiene and related diterpene olefins [103]. In a pair of paralogous Norway spruce diTPS, reciprocal exchange of four residues was sufficient to achieve the functional interconversion between a LAS- and an ISO-type diTPS, with the major contributor being an alanine residue located in the hinge region [76] (Fig. 5.6). Interestingly, while this catalytic quartet is strictly conserved among gymnosperm LAS-type diTPS, residues in ISO enzymes such as in the recently identified balsam fir ISO can differ, suggesting that formation of a tertiary abietenyl carbocation toward abietanes, rather than direct deprotonation to isopimaradiene is dependent on strict conservation of these amino acids [33]. Even more strikingly, a conserved isoleucine located directly adjacent to the hinge region was shown to determine the catalytic fidelity of *ent*-kaurene synthases in rice (*Oryza sativa*, L.), *Arabidopsis* and white spruce, while substitution of this residue resulted in formation of pimaradiene, that is, from a central intermediate in general metabolism to specialized diterpene with functions in plant defense [104–106]. Given its importance in stabilizing the intermediate allylic carbocations, it is not surprising that this short loop segment is widely conserved across class I TPS and is a key player in the evolutionary diversification of the protein family (Fig. 5.6).

Several rational protein engineering approaches targeting TPS α-domain functionality have been reported in recent years. These studies demonstrated the applicability of combinatorial site-directed mutagenesis for tailoring TPS activity and fidelity, including a decrease in undesirable byproducts [107–110]. However, this approach is cost- and labor-intensive, requiring individual functional characterization of dozens or hundreds of individual protein variants. Effective high-throughput assay systems are needed to enable high-throughput and automated screening of large TPS mutant libraries. It is also important to consider that TPS appear to have evolved toward diversity rather than catalytic efficiency and hence often represent the rate-limiting factor in terpenoid biosynthetic pathways in engineered *E. coli* or yeast systems [45, 109, 111]. Combining directed protein evolution with suitable screening systems would allow the design of TPS with higher stability and catalytic rates for balancing stoichiometry between upstream and downstream pathway modules and ultimately higher yields [107, 112]. Furthermore, pathway engineering has used gene fusions between different TPS or TPS and prenyl diphosphate synthases to successfully improve production of diterpene scaffolds [113], providing a powerful tool for enhancing metabolic channeling in engineered biosynthetic pathways.

5.7 TPS and Beyond, Challenges and Future Directions

Despite the many advances in plant and microbial engineering of terpenoid pathways, numerous constraints continue to impede economically viable product yields that can compete with conventional resources of terpenoid-based pharmaceuticals, fuels, and other fine chemicals. Accurate computational annotation of TPS functions

is substantially hampered by high sequence identity of functionally diverse enzymes. TPS characterization therefore requires laborious in vitro and in vivo analysis of each gene candidate, often limited by lack of commercially available substrates and standards.

Based on the larger catalytic landscape of terpenoid biosynthetic enzymes, metabolic engineering and synthetic biology approaches can be developed to recombine TPS and terpene-modifying enzymes, such as P450s, to produce natural and nonnatural terpenoids. Regulatory elements, such as transcription factors, tissue-specific promoters, and microRNAs, can expand the toolkit for microbial and plant engineering [114]. Other tools to explore for the metabolic engineering of terpenoids are efficient transporters for targeted sequestration or secretion of compounds that can interfere with essential processes of the living host cell. For example, the recently identified fungal ABC transporter *Gc*-ABC-G1, which acts on conifer monoterpenes in the mountain pine beetle system, could be engineered and utilized for the microbial production of terpenoids [24].

Acknowledgments We thank Karen Reid and Angela Chiang for excellent laboratory and project management support, and Kate Wilczak for reading of the manuscript. We thank Genome Canada, Genome British Columbia, Genome Alberta, Genome Quebec, and the Natural Sciences and Engineering Research Council of Canada for financial support of the research in the laboratory of JB. Some of the work discussed in this paper resulted from the Tria Project (www.thetriaproject.ca), the Treenomix Project (www.treenomix.ca), the SMarTForests Project (www.smartforests.ca) and the PhytoMetaSyn Project (www.phytometasyn.ca). We wish to thank all the participants in these projects who made our work possible. JB is a UBC Distinguished University Scholar.

References

1. Christianson DW (2008) Unearthing the roots of the terpenome. Curr Opin Chem Biol 2:141
2. Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J 66:212
3. Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408
4. Agrawal AA, Hastings AP, Johnson MT, Maron JL, Salminen JP (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338:113
5. Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4:184
6. Trapp S, Croteau R (2001) Defensive resin biosynthesis in conifers. Ann Rev Plant Physiol Plant Mol Biol 52:689
7. Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. Phytochemistry 67:2415
8. Boone CK, Aukema BH, Bohlmann J, Carroll AL, Raffa KF (2011) Efficacy of tree defense physiology varies with bark beetle population density: a basis for positive feedback in eruptive species. Can J For Res-Rev Can Rech For 41:1174
9. Bohlmann J (2012) Pine terpenoid defences in the mountain pine beetle epidemic and in other conifer pest interactions: specialized enemies are eating holes into a diverse, dynamic and durable defence system. Tree Physiol 32:943
10. Kolosova N, Bohlmann J (2012) Conifer Defense Against Insects and Fungal Pathogens. In Matyssek R, Schnyder H, Oßwald W, Ernst D, Munch JC, Pretzsch H (eds) Growth and Defence in Plants. Springer Berlin Heidelberg, p 85

11. Bohlmann J, Croteau R (1999) Diversity and variability of terpenoid defences in conifers: molecular genetics, biochemistry and evolution of the terpene synthase gene family in grand fir (*Abies grandis*). Novartis Found Symp 223:132
12. Barnett JR, Langenheim JH (2004) Plant resins: chemistry, evolution, ecology and ethnobotany. Ann Bot 93:784
13. Zulak KG, Dullat HK, Keeling CI, Lippert D, Bohlmann J (2010) Immunofluorescence localization of levopimaradiene/abietadiene synthase in methyl jasmonate treated stems of Sitka spruce (*Picea sitchensis*) shows activation of diterpenoid biosynthesis in cortical and developing traumatic resin ducts. Phytochemistry 71:1695
14. Zulak KG, Lippert DN, Kuzyk MA, Domanski D, Chou T, Borchers CH, Bohlmann J (2009) Targeted proteomics using selected reaction monitoring reveals the induction of specific terpene synthases in a multi-level study of methyl jasmonate-treated Norway spruce (*Picea abies*). Plant J 60:1015
15. Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol 129:1003
16. Miller B, Madilao LL, Ralph S, Bohlmann J (2005) Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. Plant Physiol 137:369
17. Zulak KG, Bohlmann J (2010) Terpenoid biosynthesis and specialized vascular cells of conifer defense. J Integr Plant Biol 52:86
18. Abbott E, Hall D, Hamberger B, Bohlmann J (2010) Laser microdissection of conifer stem tissues: isolation and analysis of high quality RNA, terpene synthase enzyme activity and terpenoid metabolites from resin ducts and cambial zone tissue of White spruce (*Picea glauca*). BMC Plant Biol 10:106
19. Kurz WA, Dymond CC, Stinson G, Rampley GJ, Neilson ET, Carroll AL, Ebata T, Safranyik L (2008) Mountain pine beetle and forest carbon feedback to climate change. Nature 452:987
20. Safranyik L, Carroll A, Régnière J, Langor DW, Riel WG, Shore TL, Peter B, Cooke BJ, Nealis VG, Taylor SW (2010) Potential for range expansion of mountain pine beetle into the boreal forest of North America. Can Entomol 142:415
21. DiGuistini S, Wang Y, Liao NY, Taylor G, Tanguay P, Feau N, Henrissat B, Chan SK, Hesse-Orce U, Alamouti SM, Tsui CK, Docking RT, Levasseur A, Haridas S, Robertson G, Birol I, Holt RA, Marra MA, Hamelin RC, Hirst M, Jones SJ, Bohlmann J, Breuil C (2011) Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont *Grosmannia clavigera*, a lodgepole pine pathogen. Proc Natl Acad Sci USA 108:2504
22. Hesse-Orce U, DiGuistini S, Keeling CI, Wang Y, Li M, Henderson H, Docking TR, Liao NY, Robertson G, Holt RA, Jones SJ, Bohlmann J, Breuil C (2010) Gene discovery for the bark beetle-vectored fungal tree pathogen *Grosmannia clavigera*. BMC Genomics 11:536
23. Keeling CI, Henderson H, Li M, Yuen M, Clark EL, Fraser JD, Huber DP, Liao NY, Docking TR, Birol I, Chan SK, Taylor GA, Palmquist D, Jones SJ, Bohlmann J (2012) Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. Insect Biochem Mol Biol 42:525
24. Wang Y, Lim L, DiGuistini S, Robertson G, Bohlmann J, Breuil C (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. New Phytol 197:886
25. Andersson MN, Grosse-Wilde E, Keeling CI, Bengtsson JM, Yuen MM, Li M, Hillbur Y, Bohlmann J, Hansson BS, Schlyter F (2013) Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles, *Ips typographus* and *Dendroctonus ponderosae* (Coleoptera: Curculionidae: Scolytinae). BMC Genomics 14:198
26. Erbilgin N, Colgan LJ (2012) Differential effects of plant ontogeny and damage type on phloem and foliage monoterpenes in jack pine (*Pinus banksiana*). Tree Physiol 32:946
27. Swift KAD (2004) Catalytic transformations of the major terpene feedstocks. Top Catal 27:143

28. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488:320
29. Bohlmann J, Keeling CI (2008) Terpenoid biomaterials. Plant J 54:656
30. Wilbon PA, Chu F, Tang C (2012) Progress in renewable polymers from natural terpenes, terpenoids, and rosin. Macromol Rapid Comm 34:8
31. Hillwig ML, Mann FM, Peters RJ (2011) Diterpenoid biopolymers: new directions for renewable materials engineering. Biopolymers 95:71
32. Wang J, Chen YP, Yao K, Wilbon PA, Zhang W, Ren L, Zhou J, Nagarkatti M, Wang C, Chu F, He X, Decho AW, Tang C (2011) Robust antimicrobial compounds and polymers derived from natural resin acids. Chem Commun 48:916
33. Zerbe P, Chiang A, Yuen M, Hamberger B, Hamberger B, Draper JA, Britton R, Bohlmann J (2012) Bifunctional *cis*-abienol synthase from *Abies balsamea* discovered by transcriptome sequencing and its implications for diterpenoid fragrance production. J Biol Chem 287:12121
34. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 93:2325
35. Mayor S (2011) Tree that provides paclitaxel is put on list of endangered species. Br Med J 343:7411
36. Jennewein S, Croteau R (2001) Taxol: biosynthesis, molecular genetics, and biotechnological applications. Appl Microbiol Biotechnol 57:13
37. Guerra-Bubb J, Croteau R, Williams RM (2012) The early stages of taxol biosynthesis: an interim report on the synthesis and identification of early pathway metabolites. Natl Prod Rep 29:683
38. Wilson SA, Roberts SC (2012) Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnol J 10:249
39. Jiang M, Stephanopoulos G, Pfeifer BA (2012) Downstream reactions and engineering in the microbially reconstituted pathway for Taxol. Appl Microbiol Biotechnol 94:841
40. Chiu P, Leung LT, Ko BC (2010) Pseudolaric acids: isolation, bioactivity and synthetic studies. Nat Prod Rep 27:1066
41. Son KH, Oh HM, Choi SK, Han DC, Kwon BM (2005) Anti-tumor abietane diterpenes from the cones of *Sequoia sempervirens*. Bioorg Med Chem Lett 15:2019
42. Tu WC, Wang SY, Chien SC, Lin FM, Chen LR, Chiu CY, Hsiao PW (2007) Diterpenes from *Cryptomeria japonica* inhibit androgen receptor transcriptional activity in prostate cancer cells. Planta Med 73:1407
43. Busch T, Kirschning A (2008) Recent advances in the total synthesis of pharmaceutically relevant diterpenes. Nat Prod Rep 25:318
44. Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330:70
45. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940
46. Hamberger B, Ohnishi T, Hamberger B, Séguin A, Bohlmann J (2011) Evolution of diterpene metabolism: Sitka spruce CYP720B4 catalyzes multiple oxidations in resin acid biosynthesis of conifer defense against insects. Plant Physiol 157:1677
47. Hall D, Zerbe P, Jancsik S, Quesada AL, Dullat H, Madilao LL, Yuen M, Bohlmann J (2013) Evolution of conifer diterpene synthases: diterpene resin acid biosynthesis in lodgepole pine and jack pine involves monofunctional and bifunctional diterpene synthases. Plant Physiol 161:600
48. McGarvey DJ, Croteau R (1995) Terpenoid metabolism. Plant Cell 7:1015
49. Coates RM (1976) Biogenetic-type rearrangements of terpenes. Fortschr Chem Org Naturst 33:73
50. Okada K (2011) The Biosynthesis of isoprenoids and the mechanisms regulating it in plants. Biosci Biotech Biochem 75:1219

51. Miller B, Oschinski C, Zimmer W (2001) First isolation of an isoprene synthase gene from poplar and successful expression of the gene in *Escherichia coli*. Planta 213:483
52. Beale MH (1990) The biosynthesis of C5-C20 terpenoid compounds. Nat Prod Rep 7:25
53. Christianson DW (2006) Structural biology and chemistry of the terpenoid cyclases. Chem Rev 106:3412
54. Gao Y, Honzatko RB, Peters RJ (2012) Terpenoid synthase structures: a so far incomplete view of complex catalysis. Nat Prod Rep 29:1153
55. Peters RJ (2010) Two rings in them all: the labdane-related diterpenoids. Nat Prod Rep 27:1521
56. Keeling CI, Dullat HK, Yuen M, Ralph SG, Jancsik S, Bohlmann J (2010) Identification and functional characterization of monofunctional *ent*-copalyl diphosphate and *ent*-kaurene synthases in white spruce reveal different patterns for diterpene synthase evolution for primary and secondary metabolism in gymnosperms. Plant Physiol 152:1197
57. Hayashi K-I, Kawaide H, Notomi M, Sakigi Y, Matsuo A, Nozaki H (2006) Identification and functional analysis of bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens*. FEBS Lett 580:6175
58. Mafu S, Hillwig ML, Peters RJ (2011) A novel labda-7,13-dien-15-ol-producing bifunctional diterpene synthase from *Selaginella moellendorffii*. Chembiochem 12:1984
59. Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol 170:657
60. Ro D-K, Arimura G, Lau SY, Piers E, Bohlmann J (2005) Loblolly pine abietadienol/abietadienal oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase. Proc Natl Acad Sci USA 102:8060
61. Jennewein S, Rithner CD, Williams RM, Croteau R (2003) Taxoid metabolism: taxoid 14ï¢-hydroxylase is a cytochrome P450-dependent monooxygenase. Arch Biochem Biophys 413:262
62. Chau M et al (2004) Taxol biosynthesis: molecular cloning and characterization of a cytochrome P450 taxoid 7ï¢-hydroxylase. Chem Biol 11:663
63. Nelson D, Werck-Reichhart D (2011) A P450-centric view of plant evolution. Plant J 66:194
64. Li G, Köllner TG, Yin Y, Jiang Y, Chen H, Xu Y, Gershenzon J, Pichersky E, Chen F (2012) Nonseed plant *Selaginella moellendorfii* has both seed plant and microbial types of terpene synthases. Proc Natl Acad Sci USA 109:14711
65. Birol I, Raymond A, Jackman SD, Pleasance S, Coope R, Taylor GA, Yuen MMS, Keeling CI, Brand D, Vandervalk BP, Kirk H, Pandoh P, Moore RA, Zhao Y, Mungall AJ, Jaquish B, Yanchuk A, Ritland C, Boyle B, Bousquet J, Ritland K, MacKay J, Bohlmann J, and SJM Jones (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. Bioinformatics 29:1492
66. Keeling CI, Weisshaar S, Ralph SG, Jancsik S, Hamberger B, Dullat HK, Bohlmann J (2011) Transcriptome mining, functional characterization, and phylogeny of a large terpene synthase gene family in spruce (*Picea spp*.). BMC Plant Biol 11:43
67. Aubourg S, Lecharny A, Bohlmann J (2002) Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. Mol Genet Genomics 267:730
68. Martin DM, Aubourg S, Schouwey MB, Daviet L, Schalk M, Toub O, Lund ST, Bohlmann J (2010) Functional annotation, genome organization and phylogeny of the grapevine (*Vitis vinifera*) terpene synthase gene family based on genome assembly, FLcDNA cloning, and enzyme assays. BMC Plant Biol 10:226
69. Köksal M, Jin Y, Coates RM, Croteau R, Christianson DW (2011) Taxadiene synthase structure and evolution of modular architecture in terpene biosynthesis. Nature 469:116
70. Köksal M, Hu H, Coates RM, Peters RJ, Christianson DW (2011) Structure and mechanism of the diterpene cyclase *ent*-copalyl diphosphate synthase. Nat Chem Biol 7:431
71. Zhou K, Gao Y, Hoy JA, Mann FM, Honzatko RB, Peters RJ (2012) Insights into diterpene cyclization from structure of bifunctional abietadiene synthase from *Abies grandis*. J Biol Chem 287:6840

72. Cao R, Zhang Y, Mann FM, Huang C, Mukkamala D, Hudock MP, Mead ME, Prisic S, Wang K, Lin FY, Chang TK, Peters RJ, Oldfield E (2010) Diterpene cyclases and the nature of the isoprene fold. Proteins 78:2417
73. McAndrew RP, Peralta-Yahya PP, DeGiovanni A, Pereira JH, Hadi MZ, Keasling JD, Adams PD (2011) Structure of a three-domain sesquiterpene synthase: a prospective target for advanced biofuels production. Structure 19:1876
74. Peters RJ, Flory JE, Jetter R, Ravn MM, Lee HJ, Coates RM, Croteau RB (2000) Abietadiene synthase from grand fir (*Abies grandis*): characterization and mechanism of action of the "pseudomature" recombinant enzyme. BioChemistry 39:15592
75. Martin DM, Fäldt J, Bohlmann J (2004) Functional characterization of nine Norway Spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol 135:1908
76. Keeling CI, Weisshaar S, Lin RP, Bohlmann J (2008) Functional plasticity of paralogous diterpene synthases involved in conifer defense. Proc Natl Acad Sci USA 105:1085
77. Keeling CI, Madilao LL, Zerbe P, Dullat HK, Bohlmann J (2011) The primary diterpene synthase products of *Picea abies* levopimaradiene/abietadiene synthase (PaLAS) are epimers of a thermally unstable diterpenol. J Biol Chem 286:21145
78. Gray PS, Mills JS (1964) The isolation of abienol from Canada Balsam, the oleoresin of *Abies balsamea* (L.) Mill. J Chem Soc 1:5822
79. Miyazawa M, Tamura N (2008) Characteristic odor components in the essential oil from yacón tubers (*Polymnia sonchifolia* Poepp. et Endl.). J Essent Oil Res 20:12
80. Duquesnoy E, Marongiu B, Castola V, Piras A, Porcedda S, Casanova J (2010) Combined analysis by GC (RI), GC-MS and 13C NMR of the supercritical fluid extract of *Abies alba* twigs. Nat Prod Commun 5:1995
81. Sallaud C, Giacalone C, Töpfer R, Goepfert S, Bakaher N, Rösti S, Tissier A (2012) Characterization of two genes for the biosynthesis of the labdane diterpene *Z*-abienol in tobacco (*Nicotiana tabacum*) glandular trichomes. Plant J 72:1
82. Coppen JJW, Hone GA (1995) Gum naval stores: turpentine and rosin from pine resin. In Coppen JJW, Hone GA (eds) Non-wood Forest Products 2. FAO, Rome, p. 62
83. Barrero AF, Alvarez-Manzaneda EJ, Altarejos J, Salido S, Ramos JM (1993) Synthesis of Ambrox® from ()-sclareol and (+)-cis-abienol. Tetrahedron 49:10405
84. Caniard A, Zerbe P, Legrand S, Cohade A, Valot N, Magnard JL, Bohlmann J, Legendre L (2012) Discovery and functional characterization of two diterpene synthases for sclareol biosynthesis in *Salvia sclarea* (L.) and their relevance for perfume manufacture. BMC Plant Biol 12:119
85. Schmiderer C, Grassi P, Novak J, Weber M, Franz C (2008) Diversity of essential oil glands of clary sage (*Salvia sclarea* L., *Lamiaceae*). Plant Biol 10:433
86. Schalk M, Pastore L, Mirata MA, Khim S, Schouwey M, Deguerry F, Pineda V, Rocci L, Daviet L (2012) Towards a biosynthetic route to sclareol and amber odorants. J Am Chem Soc 134:18900
87. Falara V, Pichersky E, Kanellis AK (2010) A copal-8-ol diphosphate synthase from the angiosperm *Cistus creticus* subsp. *creticus* is a putative key enzyme for the formation of pharmacologically active, oxygen-containing labdane-type diterpenes. Plant Physiol 154:301
88. Criswell J, Potter K, Shephard F, Beale MH, Peters RJ (2012) A single residue change leads to a hydroxylated product from the class II diterpene cyclization catalyzed by abietadiene synthase. Org Lett 14:5828
89. Seo S, Gomi K, Kaku H, Abe H, Seto H, Nakatsu S, Neya M, Kobayashi M, Nakaho K, Ichinose Y, Mitsuhara I, Ohashi Y (2012) Identification of natural diterpenes that inhibit bacterial wilt disease in tobacco, tomato and *Arabidopsis*. Plant Cell Physiol 53:1432
90. Renninger N, McPhee D (2008) Jet fuel additive farnesane derivatives and method of making using same. US Patent 7399323
91. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS (2011) Identification and microbial production of a terpene-based advanced biofuel. Nat Commun 2:483

92. Tracy NI, Chen D, Crunkleton DW, Price GL (2009) Hydrogenated monoterpenes as diesel fuel additives. Fuel 88:2238
93. Harvey BG, Wright ME, Quintana RL (2010) High-density renewable fuels based on the selective dimerization of pinenes. Energy Fuels 24:267
94. Bohlmann J, Crock J, Jetter R, Croteau R (1998) Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (*E*)-alpha-bisabolene synthase from grand fir (*Abies grandis*). Proc Natl Acad Sci USA 95:6756
95. Bowers WS, Fales HM, Thompson MJ, Uebel EC (1966) Juvenile hormone: identification of an active compound from balsam fir. Science 154:1020
96. Huber DPW, Philippe RN, Godard KA, Sturrock RN, Bohlmann J (2005) Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*. Phytochemistry 66:1427
97. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21:796
98. Özaydın B, Burd H, Lee TS, Keasling JD (2012) Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production. Metab Eng (in press)
99. Bokinsky G, Peralta-Yahya PP, George A, Holmes BM, Steen EJ, Dietrich J, Lee TS, Tullman-Ercek D, Voigt CA, Simmons BA, Keasling JD (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. Proc Natl Acad Sci USA 108:19949
100. Oldfield E, Lin F-Y (2012) Terpene biosynthesis: modularity rules. Angew Chem Int Ed Engl 51:1124
101. Facchini PJ, Bohlmann J, Covello PS, De Luca V, Mahadevan R, Page JE, Ro DK, Sensen CW, Storms R, Martin VJ (2012) Synthetic biosystems for the production of high-value plant metabolites. Trends Biotechnol 30:127
102. Schilmiller AL, Pichersky E, Last RL (2012) Taming the hydra of specialized metabolism: how systems biology and comparative approaches are revolutionizing plant biochemistry. Curr Opin Plant Biol 15:338
103. Wilderman PR, Peters RJ (2007) A single residue switch converts abietadiene synthase into a pimaradiene specific cyclase. J Am Chem Soc 129:15736
104. Zerbe P, Chiang A, Bohlmann J (2012) Mutational analysis of white spruce (*Picea glauca*) *ent*-kaurene synthase (PgKS) reveals common and distinct mechanisms of conifer diterpene synthases of general and specialized metabolism. Phytochemistry 74:30
105. Xu M, Wilderman PR, Peters RJ (2007) Following evolution's lead to a single residue switch for diterpene synthase product outcome. Proc Natl Acad Sci USA 104:7397
106. Morrone D, Xu M, Fulton DB, Determan MK, Peters RJ (2008) Increasing complexity of a diterpene synthase reaction with a single residue switch. J Am Chem Soc 130:5400
107. Yoshikuni Y, Ferrin TE, Keasling JD (2006) Designed divergent evolution of enzyme function. Nature 440:1078
108. O'Maille PE, Malone A, Dellas N, Andes Hess B Jr, Smentek L, Sheehan I, Greenhagen BT, Chappell J, Manning G, Noel JP (2008) Quantitative exploration of the catalytic landscape separating divergent plant sesquiterpene synthases. Nat Chem Biol 4:617
109. Leonard E, Ajikumar PK, Thayer K, Xiao WH, Mo JD, Tidor B, Stephanopoulos G, Prather KL (2010) Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. Proc Natl Acad Sci USA 107:13654
110. Aaron JA, Lin X, Cane DE, Christianson DW (2010) Structure of epi-isozizaene synthase from *Streptomyces coelicolor* A3(2), a platform for new terpenoid cyclization templates. Biochemistry 49:1787
111. Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD (2009) Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene. Metab Eng 11:13
112. Yoshikuni Y, Dietrich JA, Nowroozi FF, Babbitt PC, Keasling JD (2008) Redesigning enzymes based on adaptive evolution for optimal function in synthetic metabolic pathways. Chem Biol 15:607

113. Zhou YJ, Gao W, Rong Q, Jin G, Chu H, Liu W, Yang W, Zhu Z, Li G, Zhu G, Huang L, Zhao ZK (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 134:3234
114. Liu Q, Chen Y-Q (2010) A new mechanism in plant engineering: the potential roles of microRNAs in molecular breeding for crop improvement. Biotechnol Adv 28:301

Chapter 6
Engineering Elevated Vitamin C in Plants to Improve their Nutritional Content, Growth, and Tolerance to Abiotic Stress

Katherine A. Lisko, Siddique I. Aboobucker, Raquel Torres and Argelia Lorence

Abstract Vitamin C (L-ascorbic acid, AsA) is essential for human health; however, despite our dependency on plants as dietary sources of this nutrient, little is known about its metabolism in crops. Ascorbate protects cells and organelles from oxidative damage by scavenging reactive oxygen species that are produced in response to abiotic and biotic insults, and is also a cofactor of many enzymes, controls cell division, affects cell expansion, and is a modulator of plant senescence. Biosynthesis of AsA in plants is carried out by a complex metabolic network involving D-mannose/L-galactose, D-galacturonate, L-gulose, and *myo*-inositol as main precursors. The recent cloning of several genes that regulate AsA synthesis and recycling has facilitated the generation of transgenic plants with enhanced AsA levels, and in some cases as much as sixfold increases in AsA relative to wild-type plants have been achieved. In this review, we provide an overview of research revealing three aspects of the biochemistry of AsA that have not been fully covered elsewhere. First, we discuss the main findings of studies on feeding plant tissues with precursors as a proxy to determine which of the AsA biosynthetic pathways are operational in model and crop plants, and discuss these in the context of the forward and reverse genetic studies that support the operation of each pathway. Next, we critically discuss the consequences of elevating AsA content for plant growth, and finally we explore the effect of AsA content on plant performance under environmental stress.

Keywords Vitamin C · Ascorbic acid · Feeding studies · Abiotic stress · Stress tolerance · Phytoremediation · High throughput phenotyping · Phenomics

A. Lorence (✉)
Department of Chemistry and Physics, Arkansas State University,
P.O. Box 639, Arkansas 72467, USA
e-mail: alorence@astate.edu

K. A. Lisko · S. I. Aboobucker · R. Torres · A. Lorence
Arkansas Biosciences Institute, Arkansas State University,
P.O. Box 639, Arkansas 72467, USA

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_6

Abbreviations

L-Gal	= L-Galactose
L-GalL	= L-Galactono-1,4-lactone
D-GalUA	= D-Galacturonic acid
D-Glc	= D-Glucose
D-GlcUA	= D-Glucuronic acid
D-GlucL	= D-Glucuronolactone
L-Gul	= L-Gulose
L-GulL	= L-Gulono-1,4-lactone
D-Man	= D-Mannose
D-Fruc	= D-fructose
MI	= *Myo*-inositol
Suc	= Sucrose
AMR1	= Ascorbic acid mannose pathway regulator 1
AsA	= L-Ascorbic acid, ascorbate, vitamin C
AtERF98	= Arabidopsis ethylene responsive factor 98
AtPAP15	= Purple acid phosphatase 15 from *Arabidopsis thaliana*
DHA	= Dehydroascorbate
DHAR	= Dehydroascorbate reductase
GalDH	= L-Galactose dehydrogenase
GalUR	= D-Galacturonate reductase
GlcUR	= D-Glucuronate reductase
GLDH	= L-Galactono-1,4-lactone dehydrogenase
GLOase	= L-Gulono-1,4-lactone oxidase (a.k.a. GulLO)
GME	= GDP-mannose-3′,5′-epimerase
GNL	= Gluconolactonase
HBK3	= KNOTTED-like homeobox gene from Norway spruce
MIOX	= *myo*-Inositol oxygenase
MDHAR	= Monodehydroascorbate reductase
PMI	= Phosphomannose isomerase
PMM	= Phosphomannose mutase
VTC1	= GDP-mannose pyrophosphorylase
VTC2	= GDP-galactose phosphorylase
VTC4	= L-Galactose-1-phosphate phosphatase

6.1 Introduction

Ascorbate (L-ascorbic acid, AsA, a.k.a. vitamin C) is ubiquitous in plants, and serves a host of different functions. It protects cells and organelles from oxidative damage by scavenging reactive oxygen species (e.g., superoxide and H_2O_2), which are produced by aerobic metabolic processes such as photosynthesis and respiration or by environmental stresses like salt, drought, cold, and excess light. AsA also

participates in the regeneration of vitamin E [17] and acts as a substrate for synthesis of important organic acids (e.g., L-tartaric, L-threonic, L-glyceric, and L-oxalic acids) [24], as well as being a cofactor for enzymes involved in a diverse array of processes including flavonoid and phytohormone biosynthesis and the xanthophyll cycle [23]. There is also growing evidence that AsA participates in the regulation of cell division and elongation [48, 86], modulates flowering time and the onset of senescence [10], acts as a signaling molecule involved in plant response to environmental stresses such as ozone and pathogen attack [17], and regulates cell polarity during embryo development [15]. In short, AsA is crucial to plant health, as illustrated by the fact that no mutant completely devoid of AsA has ever been described. The *vtc2/vtc5* double mutant, a line defective in the expression of two genes in the D-mannose (D-Man)/L-galactose (L-Gal) pathway to AsA that is predicted to entirely lack ascorbate in fact suffers from very early growth arrest and is essentially nonviable [27].

Some animal species, including humans, do not synthesize AsA due to the lack of the enzyme catalyzing the last step of the biosynthetic pathway (L-gulono-1,4-lactone oxidase or GLOase, a.k.a. GulLO), and for them it thus is a vitamin. In addition to its essential roles as redox buffer in key organelles such as the mitochondria and the endoplasmic reticulum [70], vitamin C is an indispensable cofactor in the hydroxylation of proline to lysine, and therefore is essential for collagen synthesis and connective tissue integrity. A consequence of this is that vitamin C deficiency can cause scurvy, a condition characterized by hemorrhages, bleeding gums, and impaired wound healing. Vitamin C is also involved in a wide array of other crucial physiological processes in animals, including the synthesis of cytokines [90], modulation of nitric oxide synthase activity [49], oxidative protein folding and endoplasmic reticulum stress [70, 71], cell proliferation and apoptosis [25], activation of the epithelial cystic fibrosis transmembrane conductance regulator chloride channel [32], maintenance of immune homeostasis [101], promotion of iron absorption and mobilization, and tyrosine, folate and xenobiotic metabolism. In humans, diverse studies indicate that vitamin C may decrease the incidence of various diseases, including dementia [74], cancer [59, 60], stroke [104], heart disease [88], atherosclerosis [76], type 2 diabetes [44], and Charcot–Marie–Tooth disease, a hereditary peripheral neuropathy [83]. According to the 2003–2004 National Health and Nutrition Examination Survey, 7.1 % of the US population was vitamin C-deficient, and smokers, low-income people, and the elderly are among those at increased risk [89]. These data provide a clear rationale for enhancing the vitamin C content of food crops.

Despite the critical importance of AsA to plant health and human nutrition, it is only recently that scientists have succeeded in identifying pathways that lead to AsA synthesis in plants (reviewed in [12, 68]). In contrast to animals, which utilize D-glucuronate (D-GlcUA) as a precursor for AsA synthesis, plants rely on at least four alternative routes for AsA synthesis; these routes utilize myo-inositol (MI) [69], L-gulose (L-Gul) [102], D-Man/L-Gal [100], and D-galacturonate (D-GalUA) [1] as main precursors (Fig. 6.1). The recent cloning of several genes that regulate AsA synthesis and recycling has facilitated the generation of transgenic plants with

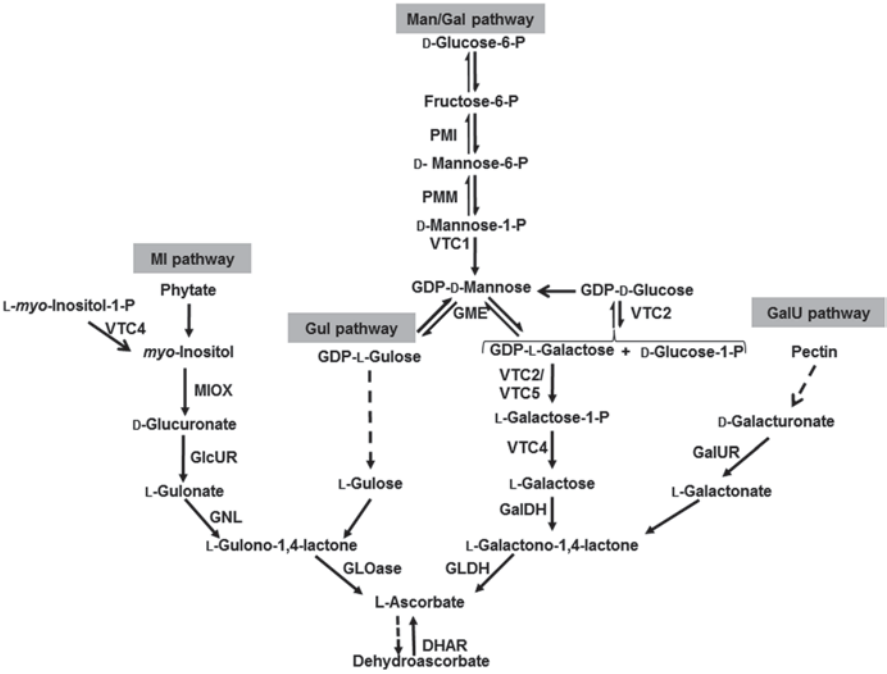


Fig. 6.1 Pathways involved in ascorbate biosynthesis and regeneration in plants: The D-mannose/L-galactose (Man/Gal) route, the L-gulose (Gul) shunt, the D-galacturonate (GalU) pathway, and the myo-inositol (MI) route. A purple acid phosphatase with phytase activity (AtPAP15) has been shown to channel phytate to the MI pathway, while VTC4 has been shown to also use L-myo-inositol-1 phosphate and contribute to both myo-inositol and ascorbate metabolisms. The enzymes participating in the Man/Gal route are: Phosphoglucose isomerase (EC 5.3.1.9); phosphomannose isomerase (PMI, EC 5.1.3.1.8); phosphomannose mutase (PMM, EC 5.4.2.8); GDP-mannose pyrophosphorylase (VTC1, EC 2.7.7.13); GDP-mannose-3′,5′-epimerase (GME, EC 5.1.3.18); GDP-galactose phosphorylase (VTC2, EC 2.7.7.B2); L-galactose-1-phosphate phosphatase (VTC4); L-galactose dehydrogenase (GalDH, EC 1.1.1.48); L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3). The enzymes in the GalU pathway are: D-galacturonate reductase (GalUR) and gluconolactonase (EC 3.1.1.17). The enzymes in the MI pathway are: Inositol phosphate phosphatase (EC 3.1.3.25); myo-inositol oxygenase (MIOX, EC 1.13.99.1); glucuronate reductase (GlcUR, EC 1.1.1.19); gluconolactonase (GNL, EC 3.1.1.17), and L-gulono-1,4-lactone oxidase (GLOase, EC 1.1.3.8). The enzymes involved in AsA recycling are: Monodehydroascorbate reductase (MDHAR, EC. 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1). Where omitted EC number have not been assigned. (Adapted from [68])

enhanced AsA levels and in some cases as much as sixfold increases in AsA relative to wild-type plants have been achieved (Table 6.1). We and various other groups who discovered these AsA biosynthetic genes have patented their use in metabolic engineering [42].

In 2004, we reported that *myo*-inositol oxygenase (MIOX) overexpression in *Arabidopsis thaliana* leads to plants with 2–3-fold increase in foliar AsA content [69]. During the past 9 years we have worked with multiple generations of these lines and, with the exception of one line where we found gene silencing, we have

Table 6.1 Successful metabolic engineering strategies that have led to high vitamin C plants

Pathway	Plant	Enzyme	Gene source	AsA fold increase	Reference
Myo-inositol	Lettuce and tobacco	GLOase	Rat	2–7	[53]
	Arabidopsis	GLOase	Rat	2–3	[87]
	Arabidopsis	MIOX4	*Arabidopsis*	2–3, N.D., 1.7	[31, 69, 94]
	Tobacco	MIOX4	*Arabidopsis*	2	[78]
	Lettuce	MIOX4	*Arabidopsis*	2–3	[78]
	Arabidopsis	AtPAP15	*Arabidopsis*	2	[105]
	Tomato	MIOX4	*Arabidopsis*	2	[56]
	Arabidopsis	GlcUR	*Arabidopsis*	2–3	Lorence et al. unpublished
D-Mannose/L-Galactose	Tobacco	GMP	Acerola	2	[6, 7]
	Arabidopsis	AMR1	*Arabidopsis*	2–3	[106]
	Tobacco	PMM	Acerola	2	[7]
	Tomato	GME	Tomato	1.6	[107]
	Tomato, potato, and strawberry	VTC2	Kiwifruit	3–6/3/2	[12]
	Arabidopsis	AtERF98	*Arabidopsis*	1.7	[108]
D-Galacturonate	*Arabidopsis*	GalUR	Strawberry	2–3	[1]
	Tomato	GalUR	Strawberry	2	[99]
	Potato	GalUR	Strawberry	2	[47]
Recycling	Tobacco and maize	DHAR	Wheat	2–4	[16]
	Tobacco	DHAR	*Arabidopsis*	2	[28]
	Potato	DHAR	Sesame	1.5	[39]
	Maize	DHAR	Rice	6	[77]
	Arabidopsis	DHAR	*Arabidopsis*	2–4	[98]
	Tomato	DHAR	Tomato	1.6	[45]
	Tomato	MDHAR	Tomato	1.27	[35]
Other pathways	Tomato	MDH	Tomato	6	[79]
	Norway Spruce	HBK3	Norway Spruce	1.6	[11]

Abbreviations of most enzyme names are described in Fig. 6.1; *AMR1* Ascorbic acid mannose pathway regulator 1; *AtERF98 Arabidopsis thaliana* ethylene response factor 98; *HBK3* KNOTTED-like homeobox gene from Norway spruce; *MDHAR* monodehydroascorbate reductase; *MDH* malate dehydrogenase; *N.D.* nondetected

always detected elevated foliar AsA content [56, 78]. In a study published in 2009, Endres and Tenhaken failed to detect differences in AsA content between a wild-type line from the Arabidopsis Stock Center in Europe and our MIOX4 overexpressors, but did detect lower MI content in those lines [31]. In 2011, a group in Hungary provided evidence and independent verification that our MIOX4 overexpressors have elevated AsA (1.7-fold), display enhanced growth (a phenotype noted by the authors and documented with a photograph in the article), and are tolerant to high light stress compared to the wild type background used for transformation [94]. We

further reported that MIOX4 over-expressors continue to have elevated AsA content (1.75-fold) compared to controls not only when grown under normal conditions, but also when subjected to salt, cold, heat, and pyrene stresses [66].

During the past few months, excellent review papers have been published describing in detail novel functions of AsA and its role in plant evolution [33, 36]. Instead of duplicating that effort, in this review we provide an overview of research revealing three aspects of the biochemistry of AsA that have not been fully covered elsewhere. First, we will discuss the main findings of studies on feeding plant tissues with AsA precursors as a proxy to determine which of the AsA biosynthetic pathways are operational in model and crop plants, and discuss these studies in the context of the forward and reverse genetic studies that support the operation of each pathway (Sect. 6.2). Next, we will critically discuss the consequences of elevating AsA content for plant growth (Sect. 6.3). Finally, also the effect of AsA content on plant performance under environmental stress will be reviewed (Sect. 6.4).

6.2 Which Biosynthetic Pathways Leading to Ascorbate Formation are Operational in Plants?

Feeding plant organs, tissues, or cells with AsA precursors has been an invaluable tool that has allowed researchers to gain a better understanding about the plasticity of the metabolic machinery leading to AsA formation in model and crop plants (Table 6.2). This approach has been employed for more than 5 decades in the field. In most cases, the experiment has consisted in the incubation of detached tissues or organs from the plant of interest in an aqueous solution of each of the AsA precursors to be tested. Most feeding experiments have been conducted under continuous light and have lasted between 1 and 24 h. The main goal of these assays has consisted in measuring the *in planta* AsA content resulting from the uptake, transport, and conversion of the substrate in question into AsA. Table 6.2 presents a summary of the studies in which more than one AsA precursor has been tested. The reader should note that we did not include several other published studies in which only one particular precursor has been fed to the plant model of study.

The results of these feeding studies should not be analyzed in isolation but as one of many tools available to understand AsA metabolism. These studies using "cold chemicals" although valuable are considered less sensitive and accurate than those where radioactively labeled precursors have been used.

Table 6.2 illustrates that without exception the AsA pathway that is operational and predominant in tissues of all the plant species analyzed with this approach is the Man/Gal route (a.k.a. Smirnoff–Wheeler pathway, [100]). Of the intermediates in this route, L-Gal is the substrate that has led to the by far highest increases in AsA, followed by L-galactono-1,4-lactone (L-GalL) with the exception of sweet pepper [9] and papaya [9] where L-GalL seems to be preferred over L-Gal. These studies also indicate that this pathway is clearly controlled by substrate availability.

Table 6.2 Feeding studies with AsA precursors in plants

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Apple (*Malus domestica)*	Flesh peel, flesh, and seed of young (y), and mature (m) fruits, 10 mM, 2 h shaking, 25 °C	Water D-Glc L-Gal L-GalL D-GalUA MI D-GlcUA L-GulL	100 (y), 100 (m) 108 (y), 109 (m) *142* (y), *132* (m) *142* (y), *127* (m) *142* (y), *145* (m) 108 (y), 91 (m) *145* (y), 118 (m) 100 (y), 100 (m)	[61]
Apple (*M. domestica*)	Leaf disks 15 mM, 30 h	Water L-Gal L-GalL L-GulL	100 *300* *300* *150*	[22]
Arabidopsis (*Arabidopsis thaliana*)	Cell suspensions (mid-log phase cells) 15 mM, 30 h 16:8 h photo-period, 24 °C	Water L-Gal L-GalL D-GalUA MI D-GlucL L-GulL	100 *6800* *3180* *3470* 100 *300* *970*	[21]
Bean (*Phaseolus vulgaris*)	Shoots (7–8 day seedlings) 0–2 %, 6–48 h	Water L-GalL D-GulL	100 *364* *157*	[8]
Black currant (*Ribes nigrum* L.)	Flowers 25 mM, 18 h shaking, dark	Water D-Glc L-Gal L-GalL D-GalUA L-Gul MI L-GulL	100 103 *296* *235* 110 108 93 108	[43]
Broccoli (*Brassica oleracea*)	Florets (petiole feeding) 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 92 *2087* *233*	[9]
Chestnut rose (*Rosa roxburghii*)	Fruit 25 mM, 24 h room temperature, natural daylight photoperiod	Water Suc D-Fruc L-GalL L-Gul Mannitol D-GlcUA	100 120 125 *200* *140* 136 125	[3]
Green sweet pepper (*Capsicum annuum*)	Whole pepper sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 86 99 *134*	[9]

Table 6.2 (continued)

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Guava (*Psidium sp.*)	Whole fruit sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 107 *158* *143*	[9]
Kiwi (*Actinidia deliciosa*)	Flesh discs 10 mM, 20 h shaking, 25 °C	Water D-Glc L-Gal L-GalL D-GalUA MI D-GlcUA L-GulL	100 *125* *162* *168* *125* 87 100 *150*	[62]
Papaya (*Carica papaya*)	Whole fruit sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 104 95 *113*	[9]
Pea (*Pisum sativum* L.)	Embryonic axes 25 mM, 8 h dark, 20 °C	Water D-Glc L-GalL L-GulL	100 102 *420* *124*	[82]
Peach (*Prunus persica* L.)	Immature whole fruits (59 days after full bloom) 25–50 mM, 18 h room temperature	Water L-Gal L-GalL D-GalUA L-GulL	100 *350* *200* 100 *125*	[51]
Rice (*Oryza sativa*)	Shoots (s) and roots (r) D-Glc 10–20 mM L-GalL 2–10 mM 72 h natural light, 25–30 °C	Water D-Glc L-GalL	100 *120* (s), *186* (r) *390* (s), *740* (r)	[40]
Strawberry (*Fragaria sp.*)	Fruit 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 103 *143* *148*	[9]
Tobacco (*Nicotiana tabacum*)	Young leaves 30 mM, 72 h 22°C, 14:10 h photoperiod light: 40 µmol m^{-2} s^{-1}	Water L-GalL L-GulL	100 *6932* *1068*	[53]
Tobacco (*N. tabacum*)	Leaf discs (young leaves) 5 mM, 6 h light: 400–1,000 µmol m^{-2} s^{-1}	Water D-Man L-Gal	100 100 *200*	[52]

Table 6.2 (continued)

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Tomato (*Solanum lycopersicum*)	Hairy root cultures 30 mM, 16–264 h 25 ± 2 °C 12 h photoperiod	Water L-Gal D-GalUA	100 *130* *210*	[99]
Tomato (*S. lycopersicum)*	Fruit disks; mature green (m) and red tomatoes (r) 15 mM, 24 h 25 °C, constant light	Water D-Glc D-Man L-Gal L-GalL MI L-GulL	100 (m), 100 (r) 124 (m), 106 (r) *130* (m), *203* (r) *246* (m), *201* (r) *287* (m), *192* (r) 112 (m), *149* (r) *180* (m), *151* (r)	[75]
Wood tobacco (*Nicotiana benthamiana*)	Detached leaves fed via petiole 50 mM, 8 h 25 °C, constant light	Water D-Man L-Gal L-Gul D-GalUA MI L-GulL	100 *132* *338* *184* 104 102 *210*	Aboobucker and Lorence, unpublished

[a] Values were normalized relative to the water control. Values in italics were reported to be significantly different from those of the water controls.
L-Gal L-Galactose; *L-GalL* L-Galactono-1,4-lactone; *D-GalUA* D-Galacturonic acid; *D-Glc* D-Glucose; *D-GlcUA* D-Glucuronic acid; *D-GlucL* D-Glucuronolactone; *L-Gul* L-Gulose; *L-GulL* L-Gulono-1,4-lactonel; *D-Man* D-Mannose; *D-Fruc* D-Fructose; *MI* Myo-inositol; *Suc* Sucrose

In addition to the results of the feeding studies listed in Table 6.2, much biochemical and molecular genetic evidence now exists in support of the Man/Gal route. Genes encoding all the proposed biosynthetic enzymes have been identified in higher plants [19, 20, 27, 34, 50, 58, 65, 72, 81, 102]. Two master regulators of this pathway, *Arabidopsis* AMR1 [106] and AtERF98 [108] have been recently reported as well.

As shown in Table 6.2, there is a significant body of work supporting the operation of alternative routes leading to AsA formation that serve to supplement the synthesis via L-Gal at certain developmental stages and in particular tissues. In the following paragraphs, we discuss the studies supporting the operation of the L-Gul, D-GalUA, and MI pathways.

Only a few of the studies have included L-Gul in the suite of precursors analyzed (Table 6.2). Of those, the one in chestnut rose [3] and the one in wood tobacco (Aboobucker and Lorence, unpublished) provided data supporting the conversion of this substrate into AsA. L-Gul does not seem to be converted into AsA in black currant under the conditions tested [43].

In addition to the feeding studies in which L-Gul has been effectively converted into AsA, molecular evidence supporting the operation of the L-Gul pathway was obtained through the characterization of the *Arabidopsis* GDP-mannose-3′-5′-epimerase (GME) enzyme [102]. GME is able to also synthesize GDP-L-gulose,

and it may be assumed that this substrate can be converted to L-gulono-1,4-lactone (L-GulL) in an analogous way to the conversion of L-Gal to L-GalL and then to AsA. Maruta and collaborators [73] obtained evidence for the operation in tobacco cells of GLOases (a.k.a. GulLOs), enzymes capable of oxidizing L-GulL into AsA. These enzymes also participate in the MI pathway to AsA.

Multiple studies carried out in apples [22], *Arabidopsis* [21], kiwi [62], and tomato fruits [107] report the conversion of D-GalUA into AsA. However, this substrate does not seem to contribute in a significant way to vitamin C synthesis in all fruits, as indicated by the lack of conversion to AsA in black currant [43], peaches [51], and leaves of wood tobacco (Aboobucker and Lorence, unpublished).

Molecular evidence in support of the operation of the GalUA pathway to AsA was provided by the characterization of an enzyme with D-GalUA reductase activity [1] in strawberry. When overexpressed, this enzyme leads to plants with elevated AsA.

Five feeding studies have included MI in the suite of precursors assayed. Of these articles, one provides convincing evidence (statistically significant differences compared to controls fed with water) of the conversion of MI into AsA in red tomatoes [75]. Inositol does not seem to be effectively converted into the final product in apples [61], *Arabidopsis* cells [21], black currant [43], or kiwi fruits [62].

Multiple teams including ours have provided molecular evidence for the operation in higher plants of enzymes capable of using MI and other inositol phosphates including phytate for AsA production [69, 93, 105]. Overexpression of AtMIOX4 led to elevated foliar AsA content in *Arabidopsis*, as demonstrated by us [69] and independently confirmed by Tóth and collaborators [94]. The enzymes that follow MIOX in the proposed MI pathway to AsA are glucuronate reductase (GlcUR), gluconolactonase (GNL), and GLOase (a.k.a. GulLO). Manuscripts with the detailed characterization of at least one member of each of these enzyme families in *Arabidopsis* are in preparation in our group.

A multitude of studies have obtained data showing the effective conversion of L-GulL into AsA in apples [22], *Arabidopsis* [21], beans [8], kiwi [62], peas [82], peaches [51], tobacco [53], tomato [75], and wood tobacco (Aboobucker and Lorence, unpublished). This substrate is structurally quite similar to the immediate AsA precursor in the Man/Gal pathway, L-GalL. However, detailed characterization of L-galactono-1,4-lactone dehydrogenase (GLDH), the terminal enzyme in that pathway, shows that plant GLDHs are highly specific for L-GalL (reviewed in [92]). Therefore, conversion of the alternative substrate L-GulL into AsA is most likely catalyzed by GulLO, the terminal enzyme that connects the L-Gul and MI pathways (Fig. 6.1).

6.3 Effects of Ascorbate Content on Plant Growth

Among the new knowledge that has emerged from the detailed characterization of the function of the various enzymes involved in AsA metabolism in plants are the remarkable negative consequences for growth, morphology, and development of lines that are deficient in this key molecule (Table 6.3). These low-AsA lines have been

Table 6.3 Effects of low ascorbate content on the phenotype, cell structure, and development of plants

Gene mutated	Plant species	Phenotype	References
VTC1	*Arabidopsis*	Reduced growth of aerial tissue Shorter primary root length Accelerated leaf senescence Delayed flowering Cells stop elongation and undergo apoptosis early in development Extensive degradation of grana stacks	[18, 80, 85, 97]
	Potato	Reduced biomass of aerial tissue accelerated leaf senescence	[54]
GME	Tomato	Reduced growth of aerial tissue reduced cell number and size reduced fruit firmness	[37]
GLDH	Rice	Reduced plant height and root length reduced leaf and root weight delayed flowering reduced number of flowers, tillers, and number of grains	[67]
	Tomato	Reduced growth of aerial tissue reduced leaf size reduced fruit diameter and weight reduced cell size	[2]
DHAR	Tobacco	Reduced rate of leaf expansion reduced growth of aerial tissue delayed flowering	[14]

developed either after chemical mutagenesis or via knockout approaches. A common phenotype reported for *Arabidopsis*, potato, rice, tomato, and tobacco low-AsA mutants is a significant reduction of growth and biomass accumulation of both aerial and root tissues. At the cellular level, this reduction in plant size and biomass is linked in some cases with decreased cell size and in others with lower number of cells. Reduced AsA levels also seem to have a negative impact on the number of flowers, number of tillers, the size of the fruits, and seed yield. On the other hand, a question that remains open is whether elevated AsA has positive effects for plant growth and development.

We reported that *Arabidopsis* lines with enhanced AsA content overexpressing enzymes that participate in the inositol pathway, MIOX4 [69] and GLOase [87], accumulate more biomass (measured as dry weight of the aerial tissue) and display a longer inflorescence stem and a wider rosette diameter compared to controls growing in soil under similar conditions. These MIOX4 and GLOase overexpressors also showed enhanced growth of both aerial and root tissues when grown in liquid culture [66, 78]. To our knowledge, this is the first study demonstrating such a marked positive effect on plant growth in lines engineered to have elevated AsA concentrations.

We have recently incorporated the use of a Scanalyzer HTS instrument (LemnaTec, Germany), a powerful tool that allows nondestructive, unbiased, and accurate phenotyping of small plants to the characterization of the high AsA lines (MIOX4 and GLOase). For this experiment, seeds of wild type (untransformed *A.*

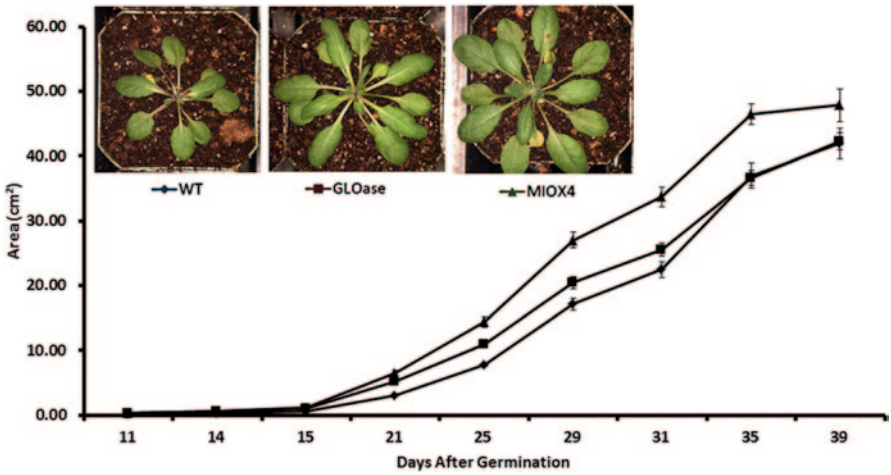


Fig. 6.2 Arabidopsis high vitamin C lines, MIOX4 and GLOase, display enhanced growth rate and biomass accumulation compared to wild-type controls. Seeds were germinated on MS media and seedlings were transferred to soil and grown under controlled conditions (23 °C, 65 % humidity, 14:10 h photoperiod, and 150 µmol m^{-2} s^{-1} light intensity). Images were acquired with a nondestructive high throughput phenotyping system (Scanalyzer HTS, LemnaTec, Germany), and leaf area was measured as previously described [4]. Images shown correspond to plants at the end of the vegetative growth (29 days after germination). Values are means ± standard error ($n = 15$)

thaliana var. Columbia, ABRC stock CS-60000) and homozygous MIOX4 and GLOase lines were cleaned and planted on Murashige and Skoog (MS) plates and vernalized for 3 days at 4 °C. Plates were then incubated in a controlled environmental chamber (Conviron, Pembina, ND) under the following conditions: 23 °C, 65 % humidity, 14:10 h photoperiod, and 150 µmol m^{-2} s^{-1} light intensity. After a week, seedlings were transferred to the soil (Arabidopsis Growing Medium, Lehle Seeds, Round Rock, TX) and grown in QuickPot 15 trays (HerkuPlast, Germany) until maturity under the above conditions. During their entire life cycle, plants were phenotyped with the Scanalyzer HTS. The acquired images were analyzed as previously described [4] to calculate plant growth (measured as foliar area in cm^2). As illustrated in Fig. 6.2, MIOX4 and GLOase overexpressors grew faster and accumulated more biomass than untransformed controls growing under similar conditions.

6.4 Effects of Ascorbate Content on Abiotic Stress Tolerance

Table 6.4 summarizes the results obtained by us and others after detailed characterization of model and crop plants with enhanced AsA content in response to various forms of abiotic stress. The AsA enhancement has been achieved in most cases after constitutive expression of biosynthetic and recycling genes; however, in some cases

Table 6.4 Stress responses of plants with elevated AsA content

Species	Method to elevate AsA	Fold AsA	Plants' performance	Reference
Arabidopsis	OE rice DHAR	1.2	Salt tolerance	[96]
	OE *Arabidopsis* DHAR	2–4.2	Paraquat tolerance Heat tolerance	[16]
	OE *Arabidopsis* MIOX4	1.7	Heat and high-light tolerance	[46]
	OE *Arabidopsis* MIOX4 and rat GLOase	1.48–1.75	Salt tolerance Cold tolerance Heat tolerance Tolerance to pyrene	[46, 66, 78]
Maize	Feeding AsA	1.25	Drought tolerance	[26]
Potato	OE rat GLOase	1.4	paraquat tolerance osmotic stress tolerance salt tolerance	[95]
	OE *Arabidopsis* DHAR	2.2–2.8	Paraquat tolerance osmotic stress tolerance salt tolerance	[30]
Rice	Feeding AsA and AsA precursors	2.9 shoots 6.4 roots	Chilling tolerance drought tolerance Al toxicity tolerance	[52]
Soybean	Feeding AsA	NM	Salt tolerance	[41]
Tobacco	OE human DHAR	No change	Cold tolerance salt tolerance	[57]
	OE wheat DHAR	2–4	ozone tolerance	[13]
	OE *Arabidopsis* DHAR	1.9–2.1	Drought tolerance ozone tolerance salt tolerance osmotic stress tolerance	[28]
	OE *Arabidopsis* MDHAR	2–2.2	Salt tolerance ozone tolerance osmotic stress tolerance	[29]
	OE *Arabidopsis* DHAR	1.3	Tolerance to Al stress	[103]
Tomato	Feeding AsA	NM	Salt tolerance	[91]
	OE tomato MDHAR	1.2	cold tolerance heat tolerance paraquat tolerance	[63]
	OE tomato GME	1.2–1.4 leaves 1.2–1.6 fruits	Paraquat tolerance cold tolerance salt tolerance	[107]
	OE potato DHAR	1.2–1.4 leaves 1.1–1.2 fruits	Paraquat tolerance salt tolerance	[64]
Wheat	Feeding AsA	1.3–1.4	Salt tolerance	[5]

NM not measured; *OE* overexpression

feeding AsA or its precursors has also led to elevated antioxidant content. The big picture that emerges from this summary is that even modest increases in AsA content have led to broad tolerance to common stresses such as salt, cold, ozone, and herbicide treatment. In addition to the overall AsA content, an aspect equally important for the health of the plant tissue is the ratio of reduced to oxidized ascorbate

or AsA redox status. In some of these studies, particularly those where DHAR and MDHAR have been overexpressed, the redox status of the plants changed, helping them overcome the stresses they have been subjected to.

Interestingly, exposure to pyrene, a polycyclic aromatic hydrocarbon and a known inducer of oxidative stress in plants, led to stunted growth of the aerial tissue, reduction in the number of root hairs, and inhibition of leaf expansion in wild-type plants, while these symptoms were less severe in the MIOX4 and GLOase overexpressors [46]. These results indicate the potential of high AsA crops as a tool for phytoremediation applications.

As AsA is intertwined in such a large number of networks (photosynthesis, flowering, ROS signaling, cell growth/division, pathogen response) and elevation of AsA levels has been shown to alter the transcription of many genes [55, 84], there could be unexpected, perhaps negative, consequences to significant elevation of AsA in plants beyond the normal physiological level. Additional research is needed in this regard.

6.5 Conclusions and Perspectives

Feeding studies have revealed that the Man/Gal pathway is operational and predominant in all the plant species analyzed to date. Of the intermediates participating in this route, L-Gal and L-GalL are most effective at increasing AsA content. However, without a doubt, these feeding studies also demonstrate the operation of all the alternative pathways that supplement the one using L-Gal in particular tissues and developmental stages. The research here reviewed indicates the potential of engineering elevated AsA content as an effective strategy to develop crops with enhanced biomass and abiotic stress tolerance. Based on our results and the ones of others, we propose that increases between 1.5- and 6-fold in the total AsA content of plant tissues are necessary to have broad tolerance to abiotic stresses in crops. Future generations of these engineered crops are likely to include the combination of the expression of biosynthetic and recycling genes, and/or the expression of regulatory proteins and transcription factors that have been recently shown to modulate multiple genes at once (e.g., [106, 108]). Although the results obtained by us and others show significant promise for the development of plants with enhanced abiotic stress tolerance, an aspect until now not fully explored is the evaluation of the consequences of elevated AsA content in the ability of plants to interact with insects and other herbivores (reviewed in [38]). This is a crucial aspect that must be evaluated at both greenhouse and field levels before these crops can be deployed.

Acknowledgments This study was supported by funds from the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000, and a sub-award to AL from the Arkansas INBRE program [National Center for Research Resources (5P20RR016460-11) and the National Institute of General Medical Sciences (8P20GM103429-11) from the National Institutes of Health]. The Scanalyzer HTS was acquired with funds from the

Arkansas Center for Plant-Powered Production (P3) through the RII Arkansas ASSET Initiative (AR EPSCoR), NSF grant # EPS 0701890. KAL and SIA thank the Molecular Biosciences Graduate program at Arkansas State University for a scholarship.

References

1. Agius F, Gonzalez-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 21:177–181
2. Alhagdow M, Mounet F, Gilbert L, Nunes-Nesi A, Garcia V, Just D, Petit J, Beauvoit B, Fernie AR, Rothan C, Baldet P (2007) Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato. Plant Physiol 145:1408–1422
3. An HM, Fan WG, Chen LG, Asghar S, Liu QL (2007) Molecular characterization and expression of L-galactono-1,4-lactone dehydrogenase and L-ascorbic acid accumulation during fruit development in *Rosa roxburghii*. J Hortic Sci Biotech 82:627–635
4. Arvidsson S, Perez-Rodriguez P, Mueller-Roeber B (2011) A growth phenotyping pipeline for *Arabidopsis thaliana* integrating image analysis and rosette area modeling for robust quantification of geno-type effects. New Phytologist. doi:10.1111/j.1469-8137.2011.03756.x
5. Athar HR, Khan A, Ashraf M (2008) Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. Environ Exp Bot 63:224–231
6. Badejo AA, Tanaka N, Esaka M (2008) Analysis of GDP-D-mannose pyrophosphorylase gene promoter from acerola (*Malpighia glabra*) and increase in ascorbate content of transgenic tobacco expressing the acerola gene. Plant Cell Physiol 49:126–132
7. Badejo AA, Eltelib HA, Fukunaga K, Fujikawa Y, Esaka M (2009) Increase in ascorbate content of transgenic tobacco plants overexpressing the acerola (*Malpighia glabra*) phosphomannomutase gene. Plant Cell Physiol 50:423–428
8. Baig MM, Kelly S, Loewus F (1970) L-Ascorbic acid biosynthesis in higher plants from L-gulono-1,4-lactone and L-galactono-1,4-lactone. Plant Physiol 46:277–280
9. Barata-Soares AD, Gomez MLPA, Henrique deMC, Lajol FM (2004) Ascorbic acid biosynthesis: a precursor study on plants. Braz J Plant Physiol 16:147–154
10. Barth C, De Tullio M, Conklin PL (2006) The role of ascorbic acid in the control of flowering time and the onset of senescence. J Exp Bot 57:1657–1665
11. Belmonte MF, Stasolla C (2009) Altered HBK3 expression affects glutathione and ascorbate metabolism during the early phases of Norway spruce (*Picea abies*) somatic embryogenesis. Plant Physiol Biochem 47:904–911
12. Bulley S, Wright M, Rommens C, Yan H, Rassam M, Lin-Wang K, Andre C, Brewster D, Karunairetnam S, Allan AC, Laing WA (2012) Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. Plant Biotechnol J 10:390–397
13. Chen Z, Gallie DR (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiol 138:1673–1689
14. Chen Z, Gallie DR (2006) Dehydroascorbate reductase affects leaf growth, development, and function. Plant Physiol 142:775–777
15. Chen Z, Gallie DR (2012) Induction of monozygotic twinning by ascorbic acid in tobacco. PLoS ONE 7. doi:10.1371/journal.pone 0039147
16. Chen Z, Young TE, Ling J, Chang S-C, Gallie DR (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Natl Acad Sci USA 100:3525–3230
17. Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ 27:959–970

18. Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci USA 93:9970–9974
19. Conklin PL, Norris SN, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc Natl Acad Sci USA 96:4198–4203
20. Conklin PL, Gatzek S, Wheeler GL, Dowdle J, Raymond MJ, Rolinski S, Isupov M, Littlechild JA, Smirnoff N (2006) *Arabidopsis thaliana vtc4* encodes L-galactose-1-P-phosphatase, a plant ascorbic acid biosynthetic enzyme. J Biol Chem 281:15662–15670
21. Davey MW, Gilot C, Persiau G, Østergaard J, Han Y, Bauw GC, Van Montagu MC (1999) Ascorbate biosynthesis in Arabidopsis cell suspension culture. Plant Physiol 121:535–543
22. Davey MW, Franck C, Keulemans J (2004) Distribution, developmental and stress responses of antioxidant metabolism in *Malus*. Plant Cell Environ 27:1309–1320
23. De Tullio MC, Arrigoni O (2004) Hopes, disillusions and more hopes from vitamin C. Cell Molec Life Sci 61:209–219
24. Debolt S, Melino V, Ford CM (2007) Ascorbate as a biosynthetic precursor in plants. Ann Bot 99:3–8
25. Dhar-Mascareño M, Cárcamo JM, Golde DW (2005) Hypoxia-reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C. Free Rad Biol Med 38:1311–1322
26. Dolatabadian A, Modarres-Sanavy SAM, Sharifi M (2009) Alleviation of water deficit stress effects by foliar application of ascorbic acid on *Zea may*s L. J Agron Crop Sci 195:347–355
27. Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N (2007) Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J 52:673–689
28. Eltayeb AE, Kawano N, Badawi H, Kaminaka H, Sanekata T, Morishima I, Shibahara T, Inanaga S, Tanaka K (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. Physiol Plant 127:57–65
29. Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt, and polyethylene glycol stresses. Planta 225:1255–1264
30. Eltayeb AE, Yamamoto S, Eltayeb Habora ME, Yin L, Tsujimoto H, Tanaka K (2011) Transgenic potato overexpressing Arabidopsis cytosolic *AtDHAR1* showed higher tolerance to herbicide, drought, and salt stresses. Breeding Sci 61:3–10
31. Endres S, Tenhaken R (2009) *Myo*-inositol oxygenase controls the level of *myo*-inositol in Arabidopsis, but does not increase ascorbic acud. Plant Physiol 149:1042–1049
32. Fischer H, Schwarzer C, Illek B (2004) Vitamin C controls the cystic fibrosis transmembrane conductance regulator chloride channel. Proc Natl Acad Sci USA 101:3691–3696
33. Gallie DR (2013) The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. J Exp Bot 64:433–443
34. Gatzek S, Wheeler GL, Smirnoff N (2002) Antisense supression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate biosynthesis and reveals light modulated L-galactose synthesis. Plant J 30:541–553
35. Gest N, Garchery C, Gautier H, Jimenez A, Stevens R (2013) Light-dependent regulation of ascorbate in tomato by a monodehydroascorbate reductase localized in peroxisomes and the cytosol. Plant Biotech J 11:344–354
36. Gest N, Gautier H, Stevens R (2013) Ascorbate as seen through plant evolution: the rise of a successful molecule? J Exp Bot 64:33–53
37. Gilbert L, Alhagdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V, Petit J, Stevens R, Causse M, Fernie AR, Lahaye M, Rothan C, Baldet P (2009) GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell wall biosynthesis in tomato. Plant J 60:499–508
38. Goggin FL, Avila CA, Lorence A (2010) Vitamin C content in plants is modified by insects and influence susceptibility to herbivory. Bioessays 32:777–790

39. Goo YM, Chun HJ, Kim TW, Lee CH, Ahn MJ, Bae SC, Cho KJ, Chun JA, Chung CH, Lee SW (2008) Expressional characterization of dehydroascorbate reductase cDNA in transgenic potato plants. J Plant Biol 51:35–41
40. Guo Z, Tan H, Zhu Z, Lu S, Zhou B (2005) Effect of intermediates on ascorbic acid and oxalate biosynthesis of rice in relation to its stress resistance. Plant Physiol Biochem 43:955–962
41. Hamada AM, Al-Hakimi ABM (2009) Hydroponic treatment with ascorbic acid decreases the effect of salinity injury in two soybean cultivars. Phyton 49:43–62
42. Hancock R (2009) Recent patents on vitamin C: opportunities for crop improvement and single-step biological manufacture. Rec Patents Food Nutr Agric 1:39
43. Hancock RD, Walker PG, Pont SDA, Marquis N, Vivera S, Gordon SL, Brennan RM, Viola R (2007) L-Ascorbic acid accumulation in fruit of *Ribes nigrum* occurs by *in situ* biosynthesis via the L-galactose pathway. Funct Plant Biol 34:1080–1091
44. Harding A-H, Wareham NJ, Bingham SA, Khaw K, Luben R, Welch A, Forouhi NG (2008) Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: the european prospective investigation of cancer-Norfolk prospective study. Arch Intern Med 168:1493–1499
45. Haroldsen VM, Chi-Ham CL, Kulkarni S, Lorence A, Bennett AB (2011) Constitutively expressed DHAR and MDHAR influence fruit, but not foliar ascorbate levels in tomato. Plant Physiol Biochem 49:1244–1249
46. Harris RS (2009) Analysis of the protective effects of ascorbic acid on thrichloroethylene and pyrene phytotoxicity. Master's Thesis. Arkansas State University, Jonesboro, AR, USA
47. Hemavathi, Upadhyaya CP, Young KE, Akula N, Kim HS, Heung JJ, Oh OM, Aswath CR, Chun SC, Kim DH, Park SW (2009) Over-expression of strawberry D-galacturonic acid reductase in potato leads to accumulation of vitamin C with enhanced abiotic stress tolerance. Plant Sci 177:659–667
48. Horemans N, Foyer CH, Potters G, Asard H (2000) Ascorbate function and associated transport systems in plants. Plant Physiol Biochem 38:531–540
49. Huang A, Vita JA, Venema RC, Keaney Jr JF (2000) Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. J Biol Chem 275:17399–17406
50. Imai T, Karita S, Shiratori G, Hattori M, Numome T, Oba K, Hirai M (1998) L-Galactono-ɣ-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. Plant Cell Physiol 39:1350–1358
51. Imai T, Ban Y, Terakami S, Yamamoto T, Moriguchi T (2009) L-ascorbate biosynthesis in peach: cloning of six L-galactose pathway-related genes and their expression during peach fruit development. Physiol Plant 136:139–149
52. Imai T, Ban Y, Yamamoto T (2012) Ectopic overexpression of peach GDP-D-mannose pyrophosphorylase and GDP-D-mannose-3',5'-eperimase in transgenic tobacco. Plant Cell Tiss Organ Cult 111:1–13
53. Jain AK, Nessler CL (2000) Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. Mol Breeding 6:73–78
54. Keller R, Springer F, Renz A, Kossmann J (1999) Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. Plant J 19:131–141
55. Kiddle G, Pastori GM, Bernard S, Pignocchi C, Antoniw J, Verrier PJ, Foyer CH (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in Arabidopsis thaliana. Antiox Redox Signal 5:23–32
56. Kulkarni S (2012) Elevating ascorbate content in tomato and studying the role of jasmonates in modulating ascorbate in Arabidopsis. MS Thesis, Arkansas State University, Jonesboro, AR, USA
57. Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, Kwak SS (2003) Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. J Plant Physiol 160:347–353

58. Laing WA, Wright MA, Cooney J, Bulley SM (2007) The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc Natl Acad Sci USA 104:9534–9539
59. Lee KW, Lee HJ, Surh YJ, Lee CY (2003) Vitamin C and cancer chemo- prevention: reappraisal. Am J Clin Nutr 78:1074–1078
60. Levi F, Pasche C, Lucchini F, La Vecchia C (2001) Dietary intake of selected micro- nutrients and breast-cancer risk. Int J Cancer 91:260–263
61. Li MJ, Ma FW, Zhang M, Pu F (2008) Distribution and metabolism of ascorbic acid in apple fruits (*Malus domestica* Borkh cv. Gala). Plant Sci 174:606–612
62. Li M, Ma F, Liang D, Li J, Wang Y (2010) Ascorbate biosynthesis during early fruit development is the main reason for its accumulation in kiwi. PLoS ONE 5. doi: 10.1371/journal.pone.0014281
63. Li F, Wu QY, Sun YL, Wang LY, Yang XH, Meng QW (2010) Overexpression of chloroplastic monodehydroascorbate reductase enhance tolerance to temperature and methyl viologen-mediated oxidative stress. Physiol Plant 139:421–434
64. Li Q, Li Y, Li C, Yu X (2012) Enhanced ascorbic acid accumulation through overexpression of dehydroascorbate reductase confers tolerance to methyl viologen and salt stresses in tomato. Czech J Genet Plant Breed 48:74–86
65. Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Clarke SG (2007) Arabidopsis *VTC2* encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J Biol Chem 282:18879–18885
66. Lisko KA, Torres R, Harris RS, Belisle M, Jullian B, Vaughan MM, Chevone BI, Mendes P, Nessler CL, Lorence A (2013) Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulono-1,4-lactone oxidase in Arabidopsis leads to enhanced biomass and tolerance to abiotic stresses. In Vitro Cel Develop Biol Plant49(6):643–655
67. Liu Y, Yu L, Wang R (2011) Level of ascorbic acid in transgenic rice for L-galactono-1,4-lactone dehydrogenase overexpressing or suppressed is associated with plant growth and seed set. Acta Physiol Plant 33:1353–1363
68. Lorence A, Nessler CL (2007) Pathway engineering of the plant vitamin C metabolic network. In: Verpoorte R, Alferman AW, Johnson TS (eds) Applications of plant metabolic engineering. Springer, Dordrecht
69. Lorence A, Chevone BI, Mendes P, Nessler CL (2004) *myo*-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol 134:1200–1205
70. Mandl J, Szarka A, Bánhegyi G (2009) Vitamin C: update on physiology and pharmacology. British J Pharmacol 157:1097–1110
71. Margittai E, Banhegyi G, Kiss A, Nagy G, Mandl J, Schaff Z, Csala M (2005) Scurvy leads to endoplasmic reticulum stress and apoptosis in the liver of guinea pigs. J Nutr 135:2530–2534
72. Maruta T, Yonemitsu M, Yabuta Y, Tamoi M, Ishikawa T, Shigeoka S (2008) Arabidopsis phosphomannose isomerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid biosynthesis. J Biol Chem 283:28842–28851
73. Maruta T, Ichikawa Y, Mieda T, Takeda T, Tamoi M, Yabuta Y, Ishikawa T, Shigeoka S (2010) The contribution of Arabidopsis homologs of L-gulono-1,4-lactone oxidase to the biosynthesis of ascorbic acid. Biosci Biotechnol Biochem 74:1494–1497
74. Masaki KH, Losonczy KG, Izmirlian G, Foley DJ, Ross GW, Petrovitch H, Havlik R, White LR (2000) Association of vitamin E and C supplement use with cognitive function and dementia in elderly men. Neurology 54:1265–1272
75. Mellidou I, Keulemans J, Kanellis AK, Davey MW (2012) Regulation of fruit ascorbic acid concentrations during ripening in high and low vitamin C tomato cultivars. BMC Plant Biol 12:239
76. Napoli C, Williams-Ignarro S, de Nigris F, Lerman LO, Rossi L, Guarino C, Mansueto G, Di Tuoro F, Pignalosa O, De Rosa G, Sica V, Ignarro LJ (2004) Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice. Proc Natl Acad Sci USA 101:8797–8802
77. Naqvi S, Zhu CF, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortifica-

tion of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767
78. Nessler CL, Lorence A, Chevone B, Mendes P (2007) Increase in plant growth rate, biomass accumulation and stress tolerance in plants over-expressing genes of the ascorbic acid-cell wall biosynthetic network. US Patent Application No 11/908, 551
79. Nunes-Nesi A, Carrari F, Lytovchenko A, Smith AM, Loureiro ME, Ratcliffe AG, Sweetlove LJ, Fernie AR (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol 137:611–622
80. Olmos E, Kiddle G, Pellny TK, Kumar S, Foyer CH (2006) Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. J Exp Bot 57:1645–1655
81. Ostergaard J, Persiau G, Davey MW, Bauw G, van Montagu G (1997) Isolation of a cDNA coding for L-galactono-ɣ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. J Biol Chem 272:30009–30016
82. Pallanca JE, Smirnoff N (1999) Ascorbic acid metabolism in pea seedlings. A comparison of D-glucosone, L-sorbosone, and L-galactono-1,4-lactone as ascorbate precursors. Plant Physiol 120:453–461
83. Passage E, Norreel JC, Noack-Fraissignes P, Sanguedolce V, Pizant J, Thirion X, Robaglia-Schlupp A, Pellissier JF, Fontés M (2004) Ascorbic acid treatment corrects the phenotype of a mouse model of Charcot-Marie-Tooth disease. Nat Med 10:396–401
84. Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 15:939–951
85. Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniw J, Alvarez ME, Foyer CH (2005) Ascorbic acid deficiency activates cell death and disease resistance in *Arabidopsis thaliana*. Plant Physiol 139:1291–1303
86. Potters G, De Gara L, Asard H, Hoemans N (2002) Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol Biochem 40:537–548
87. Radzio JA, Lorence A, Chevone BI, Nessler CL (2003) L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient Arabidopsis (*vtc*) mutants. Plant Molec Biol 53:837–844
88. Rinne T, Mutschler E, Wimmer-Greinecker G, Moritz A, Olbrich HG (2000) Vitamins C and E protect isolated cardiomyocytes against oxidative damage. Int J Cardiol 75:275–281
89. Schleicher RL, Carroll MD, Ford ES, Lacher DA (2009) Serum vitamin C and the prevalence of vitamin C deficiency in the United States: 2003–2004 National Health and Nutrition Examination Survey (NHANES). Am J Clin Nutr 90:1252–1263
90. Schwager J, Schulze J (1998) Modulation of interleukin production by ascorbic acid. Vet Immunol Immunopathol 64:45–57
91. Shalata A, Neumann PM (2001) Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduced lipid peroxidation. J Exp Bot 52:2207–2211
92. Smirnoff N (2011) Vitamin C: the metabolism anf functions of ascorbic acid in plants. Adv Bot Res 59:107–177
93. Torabinejad J, Donahue JL, Gunesekera BN, Allen-Daniels MJ, Gillaspy GE (2009) *VTC4* is a bifunctional enzyme that affects *myo*-inositol and ascorbate biosynthesis in plants. Plant Physiol 150:951–961
94. Tóth SZ, Nagy V, Puthur JT, Kovacs L, Garab G (2011) The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. Plant Physiol 156:382–392
95. Upadhyaya HCP, Akula N, Young KE, Young KE, Chun SC, Kim DH, Park SW (2010) Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. Biotechnol Lett 32:321–330
96. Ushimaru T, Nakagawa T, Fujioka Y, Daicho K, Naito M, Yamauchi Y, Nonaka H, Amako K, Yamawaki K, Murata N (2006) Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. J Plant Physiol 163:1179–1184

97. Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH (2001) Low ascorbic acid in the *vtc*-1 mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. Plant Physiol 127:426–435
98. Wang Z, Xiao Y, Chen W, Tank K, Zhang L (2010) Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis. JIPB 52:400–409
99. Wevar-Oller AL, Agostini E, Milrad SR, Medina MI (2009) In situ and de novo biosynthesis of vitamin C in wild type and transgenic tomato hairy roots: a precursor feeding study. Plant Sci 177:28–34
100. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369
101. Wintergerst ES, Maggini S, Hornig DH (2006) Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. Ann Nutr Metab 50:85–94
102. Wolucka BA, Van Montagu M (2003) GDP-mannose-3'-5'-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosyn thesis of vitamin C in plants. J Biol Chem 278:47483–47490
103. Yin L, Wanf S, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, Takeuchi Y, Tanaka K (2010) Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta 231:609–621
104. Yokoyama T, Date C, Kokubo Y, Yoshiike N, Matsumura Y, Tanaka H (2000) Serum vitamin C concentration was inversely associated with subsequent 20-year incidence of stroke in a japanese rural community: the Shibata study. Stroke 31:2287–2294
105. Zhang W, Gruszewski HA, Chevone BI, Nessler CL (2008) An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 146:431–440
106. Zhang WY, Lorence A, Gruszewski HA, Chevone B, Nessler CL (2009) *AMR1*, an Arabidopsis gene that coordinately and negatively regulates the D-mannose/L-galactose ascorbic acid biosynthetic pathway. Plant Physiol 150:942–950
107. Zhang C, Liu J, Zhang Y, Cai X, Gong P, Zhang J, Wang T, Li H, Ye Z (2011) Overexpression of SlGMEs leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. Plant Cell Rep 30:389–398
108. Zhang Z, Wang J, Zhang R, Huang R (2012) The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in Arabidopsis. Plant J 71:273–287

Chapter 7
Evaluating the Phytochemical Potential of Camelina: An Emerging New Crop of Old World Origin

Mark A. Berhow, Steven F. Vaughn, Bryan R. Moser, Deniz Belenli and Umit Polat

Abstract Out on the next frontier of nutritional research will be the complete biochemical and physiological characterization of plant-derived foods that prevent or delay the development of chronic diseases in humans and animals. The chemical composition of many major crop products (seeds, flour, oil, leaves, etc.) have been determined, but the slow process of evaluating each compound alone or in mixtures for the biological function in nutrition and health of the animals that consume them has only just begun. Camelina, or false flax (*Camelina sativa* L. Crantz), is an emerging oil seed crop in North America mostly used as a biodiesel fuel. The seeds contain up to 45% oil, which is rich in polyunsaturated omega-3 and omega-6 fatty acids, as well as fat-soluble antioxidants such as the vitamin E-active tocopherols. Extraction of oil from camelina seeds by mechanical expeller yields a seed meal that consists of approximately 10% residual oil, 45% crude protein, 10% soluble sugars, 13% fiber, 5% minerals, and 10% phytochemical constituents such as glucosinolates, flavonols, lignans, phenolic acids as well as nucleic acids. The seed meal also contains a hydrophilic gum. While the oil fraction has been well characterized and its uses are growing, the seed meal has yet to be fully characterized for its potential use

---

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

M. A. Berhow (✉) · S. F. Vaughn
United States Department of Agriculture, Agricultural Research Service,
National Center for Agricultural Utilization Research, Functional Foods Research Unit,
1815 N. University Street, Peoria, Illinois 61604, USA
e-mail: mark.berhow@ars.usda.gov

B. R. Moser
United States Department of Agriculture, Agricultural Research Service,
National Center for Agricultural Utilization Research, Bio-Oil Research Unit,
1815 N. University Street, Peoria, Illinois 61604, USA

D. Belenli · U. Polat
Faculty of Veterinary Medicine, Department of Biochemistry,
University of Uludag, 16059 Bursa, Turkey

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application*,
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_7

in animal feeds or in foods for humans. The phytochemical components of camelina potentially have strong benefits for use in functional food roles.

Keywords Camelina · Oil seed · Chemical composition · Glucosinolate · Flavonols · Phenolics

7.1 Complete Characterization of Plant Crop Products

The next frontier of nutritional research will be the characterization of plant-derived chemical components—and the resulting biochemical processes that they regulate in animals—that are critical in preventing or delaying the development of chronic diseases such as cancer, diabetes, heart and circulatory diseases, and other chronic conditions that develop in humans and animals. Seeds, leaves, bark, stems and flowers, as well as extracts from these plant organs, have historically been used to treat diseases and infirmaries in humans and animals. The development of the modern pharmaceutical industry owes its very existence to the characterization and purification of physiologically active chemical compounds from plants, fungi, and bacteria. Epidemiological observations have shown that regular consumption of a number of specific foods or plant extracts significantly reduces the occurrence, or slows the development of nearly all of the chronic disease conditions that afflict humankind.

The challenge in preventing chronic disease by nutritional phytochemicals is that their effect may not be due to a single chemical agent or even a set of chemically related compounds, unlike pharmacological chemicals which tend to specifically destroy disease-causing microbes or abnormal mammalian cell types. Disease prevention through nutrition may be the result of an optimal mix of phytochemical agents—from a single plant species or even a mixture of plant species—combined with a defined caloric intake and regular exercise that will result in the prevention or slowing of the development of chronic disease. Traditional Chinese and Indian medicines have been prescribed for exactly these purposes for thousands of years, yet we still do not understand how they work on a chemical or biochemical level. In order to fully understand these very complex processes, it is essential to have a complete chemical profile of the components that make up a particular food. As many of these compounds are produced by plants to mediate ecophysiological stress, microbial, or herbivore pressure, this would have the added benefit of being able to better evaluate the full chemical profile of particular plant species for more effective pest control.

Full chemical characterization of a given plant material is still a difficult endeavor even with all the advances in modern computers and analytical equipment. Also, the considerable variation in plant organ chemical composition due to genetic and environmental factors makes this complex analysis even more challenging. Analytical research has yielded fairly complete chemical characterization of the major food crops, especially the grains and soybean. These foods are fairly complex mixtures of primary metabolites and phytochemicals of which the minor components may

play key roles in determining the long-term bioactivity. Yet even in these major crops, very few of the minor phytochemical components are available as pure standards for further nutritional evaluation. Often, as in the case with soy, researchers have focused on just one family of phytochemicals, such as the isoflavones, and the resulting nutritional and disease research studies conducted on these compounds have often shown that isolated soy isoflavones are generally less effective than the whole foods.

It would be beneficial to have a relatively simple phytochemical model food system as an effective tool to use for compositional nutritional studies, such as *Arabidopsis* is being used in plant genetic studies. The nutritional model plant needs to be in crop production, as no rare and hard to obtain plant will be effective for such studies. One candidate is *Camelina sativa*, or simply camelina, which is an emerging bio-oil crop in North America from the Brassicaceae family (see Sect. 7.2) The aim of this review is to summarize all of the known chemical components of camelina seed meals with special emphasis on the analysis and determination of the phytochemical composition, with the aim of assessing the key chemical components to be evaluated for their roles in the prevention of chronic disease in animals. The oil composition has already been well characterized, so it will only be summarized here (Sect. 7.3), followed by a detailed discussion of the phytochemical compositions of camelina seed meal (Sect. 7.4) and the methods used for the analyses (Sect. 7.5).

7.2 Camelina: An Emerging New Crop in North America

Camelina sativa L. Crantz—also known as gold-of-pleasure, false flax, wild flax, linseed dodder, German sesame, and Siberian oilseed—is an annual member of the Brassicaceae (mustard family). It is a plant native to temperate northern Europe and central Asia, but was introduced to North America, possibly as a contaminant in flax seed. It was traditionally cultivated in Europe to produce vegetable oil and animal feed [1, 2]. There is ample archeological evidence to show that it has been grown for at least 3,000 years [3, 4]. Camelina was an important oil crop in eastern and central Europe, and has continued to be cultivated in a few parts of Europe for its seed, which was used in oil lamps and as edible oil. Camelina has a number of agronomic advantages: it can be grown in a variety of climatic and soil conditions as a spring/summer crop or as a biannual winter crop, and can be easily incorporated into crop rotations; it has a short growing season (85–100 days); it is compatible with existing farm practices and does not require specialized harvesting equipment; it tolerates cold weather, drought, and low-fertility/saline soils; it has few natural pests and so requires relatively little pesticide application [5–7]. It is currently grown in the northern USA and in Canada, and the USDA, AgCanada, several US states and Canadian provincial agencies, and at least two private companies have both breeding and genetic modification programs aimed at further improving camelina traits such as improved oil content, seed viability, expanded growing locations, and resistance to disease, pest, and weed competition.

Table 7.1 Yield of camelina compared to other commodity oil crops

Crop	Seed yield (kg ha-1 year-1)	Seed oil (mass %)	Oil yield (kg ha-1 year-1)
Camelina	1,500–3,000	28–47	420–1,410
Rapeseed	2,680–3,390	40–44	965–1,342
Soybean	2,140–2,840	18–22	347–562
Sunflower	1,440–1,700	39–49	505–750

Data for seed yield of rapeseed, soybean, and sunflower were taken from Oil World Annual 2009, 1, Oilseeds pp. 5–9. Data for seed oil content of camelina (and seed yield), rapeseed, soybean, and sunflower were taken from the 3rd edition of The Lipid Handbook.

Although camelina is harvested primarily for its seed, the rest of the plant could be used as a straw or as a source of cellulose and lignin [6]. Interest in the use of camelina as a functional food and as a source of biodiesel continues to grow. The seed is generally processed by cold pressing to remove 80–90 % of the oil, yielding crude oil and seed meal. Methods have been developed to refine the oil for both food and fuel uses, and the chemical composition has been extensively studied. Yields are anywhere from 336 to 2,240 kg per hectare with lipid contents of 25–45 weight percent (Table 7.1 and [8–10]). Oil yields are comparable to rapeseed, soybean, and sunflower. The interest in North America is partly due to its exceptionally high level of omega-3 fatty acids, which is uncommon in commodity vegetable sources. Over 50 % of the fatty acids in cold-pressed camelina oil are polyunsaturated (Table 7.2). Because of its apparent health benefits and its relative oil stability, camelina oil should be added to the growing list of functional foods. However, additional uses are still needed for processing coproducts to render camelina economically viable. Defatted camelina seed meal contains significant levels of proteins and carbohydrates as well as a number of phytochemicals including glucosinolates, which could be utilized in additional food, feed, and agricultural uses.

On a compositional level, a wealth of information has recently become available on the chemical components of camelina seeds. Applying modern analytical techniques can solve a few of the missing pieces of the phytochemical puzzle. In the next step, nearly all of the camelina chemical components can then be assembled to create artificial food that very closely resembles that of camelina oil and seed meal. Nutritional researchers will thus be able to evaluate the artificial mixture against natural camelina seed meal and further evaluate each of the components—either individually or as mixtures—to characterize its nutritional bioactivity.

7.3 Camelina Seed Oil

Crude camelina oil consists of about 45 % polyunsaturated fatty acids, 35 % monounsaturated fatty acids, 10 % saturated fatty acids, and up to 10 % free fatty acids, tocopherols, sterols, other terpenes and volatiles.

Table 7.2 Fatty acid composition of camelina oil ([a][16], [b][18])

	Carbon number	Weight percent[a]
Unsaturated [a, b]		
α-Linolenic acid	18:3	37%
Linoleic acid	18:2	15%
Oleic acid	18:1	15%
11Z-Eicosenoic acid	20:1	15%
Erucic acid	22:1	3%
cis-11,14-Eicosadienoic acid	20:2	2%
Eicosatrienoic acid	20:3	2%
Saturated [a, b]		
Palmitic acid	16:0	5%
Stearic acid	18:0	3%
Arachidic acid	20:0	1%
Minor[b]		
Total		2%
13Z,16Z-Docosadienoic acid	22:2	Trace
10Z-Heptadecenoic acid	17:1	0.5%
Palmitoleic acid	16:1	Trace
Nervonic acid	24:1	Variable
Heptadecanoic acid	17:0	0.2%
Heneiosanoic acid	21:0	Trace
Behenic acid	22:0	0.5%
Tricosanoic acid	23:0	Trace
Lignoceric acid	24:0	Trace

7.3.1 Camelina Oil Composition

The average concentrations are covered in detail in several published reports [9–22] and summarized in Table 7.2. α-Linolenic acid, an omega-3 fatty acid, is the most abundant fatty acid in camelina oil along with linoleic, oleic, and 11-eicosenoic acids. These fatty acids account for 80–85% of the oil. Camelina oil contains relatively high levels of erucic acid, but the amount is below the 5% threshold that is critical for food use [20, 23]. Extensive breeding programs are currently underway hope to lower the levels of this fatty acid in future crop lines.

The extractable oil fraction (Table 7.3 and [11, 16–18, 24]) includes the nonvolatile terpenes—sterols, tocopherols, small amounts of the un-cyclized terpenes squalene and phytol, and a few other degradation products—the levels of which depend on the amount of processing conducted on the sample. Of interest here, are the relatively high levels (for a plant) of cholesterol [25]. The tocopherol levels are relatively low compared to oils of other species such as soybean. The major tocopherols found in camelina are α-tocopherol, γ-tocopherol, and δ-tocopherol, with small amounts of β-tocopherol also identified. Tocotrienols have not generally been detected in camelina.

Small amounts of free fatty acids in the range of less than 0.1–0.8% are present in the extracted oil, which are probably released during the course of oil extraction, processing, and storage [16]. All plant seed oil fractions have a unique and often distinct

Table 7.3 Composition of unsaponifiables (sterols and nonvolatile terpenoids) comprising 0.5–0.8 weight percent of camelina oil ([a][16], [b][17], [c][24], [d][18])

Sterols	Tocopherols
ß-Sitosterol[a b c]	α-Tocopherol[a c]
Campesterol[a b c]	β-Tocopherol (trace)[a c]
Campestanol[c]	γ-Tocopherol[a c]
Cholesterol[a b c]	δ-Tocopherol (trace)[a c]
Brassiciasterol[a b c]	Retinoic acid (trace)[a]
γ-Sitosterol[a c]	Plastochromanol[d]
Sitostanol[c]	
δ-5-Avenosterol (trace)[a b]	Non-cyclized terpenes
δ-7-Avenosterol (trace)[c]	Squalene[a]
14-Methylergost-8-en-3-ol (trace)[a]	Phytol[a]
Stigmasterol (trace)[a]	Squalane (trace) [a]
Stigmasta-5,24-dienol[c]	
Obtusifoliol (trace)[a]	
Cycloartenol (trace)[bc]	
24-Methylenecycloartanol[c]	
α-1-Sitosterol (trace)[a]	
14-Methylfecosterol (trace)[a]	
6-Methylcholest-5-en-ol (trace)[a]	
Gramisterol + α-amyrin[c]	
β-Amyrin (trace)[a]	
Citrostadienol[c]	

odor made up of the volatile and semi-volatile compounds. Typically, this comprises a large number of alkyl and benzyl compounds, each present in relatively small amounts. The amounts and types of these compounds can vary considerably from cultivar to cultivar and depend on growth conditions, as well as the amount and type of processing in the oil extraction and preparation. Table 7.4 lists 30 volatile compounds found in freshly prepared camelina oil by headspace analysis, another reference identified 168 acids, alcohols, esters, ketones, aldehydes, alkanes, alkenes, aromatics, ethers, pyrazines, terpenes, and sulfur-containing compounds [16, 19]. Overall, this mixture of volatiles constitutes a very small fraction of the camelina oil, and their concentration in the oil continually decreases with storage time and at elevated temperatures.

7.3.2 Camelina Oil Uses

The unusually high content of polyunsaturated omega-3 fatty acids makes camelina oil useful as a nutritional food and for cosmetic applications although this has been underexploited so far.

The rise in production of camelina in North America has been fueled, so to speak, by its potential use as a feedstock for the production of biodiesel [6–8, 13–15]. Camelina oil has been successfully converted to biodiesel by a variety of catalytic and heating methods. The fuel properties are similar to those of biodiesel prepared

Table 7.4 Volatile compounds in camelina oil listed by retention time on a nonpolar GC column (less than 0.5 weight percent of fresh crude extracted oil) [19]

Compound	Compound
Acetic acid	*trans*-2-Heptenal
Ethylacetate	Benzaldehyde
trans-2-Butenyl	Sabinene
trans-3-Penten-2-one	1-Octen-3-ol
trans-2-Pentenal	6-Methyl-5-hepten-2-one
Butyric acid	β-Myrcene
Isovaleric acid	*trans, trans*-2,4-Heptadienal
trans-2-Hexenal	Octanal
Hexanol	3-Carene
2-Heptanone	p-Cymene
Styrene	Limonene
Heptenal	*trans*-3-Octen-2-one
trans, trans-2,4-Hexadienal	*trans, trans*-3,5-Octadiene-2-one
γ-Butyrolactone	Nonal
α-Pinene	Decanal

from soybean oil, but as camelina oil contains a high percentage of polyunsaturated fatty acid methyl esters, it requires antioxidant additives to meet fuel stability specifications, which is typical for most biodiesels. Camelina-based diesel blends provide fuel performance characteristics similar to those of the corresponding soybean-based blends. Camelina oil can also be converted to a wax ester that can be used as a biolubricant and an ingredient for cosmetics [6, 7].

7.4 Camelina Seed Meal

Extraction of the oil from camelina seeds is typically done by mechanical expellers which yield a seed meal that consists of approximately 10% residual oil, 45% protein, up to 15% carbohydrate/lignin insoluble fiber, up to 10% soluble carbohydrates, 5% minerals, approximately 0.2% nucleic acids, and 10% or more of a mixture of phytochemical components consisting mostly of glycosylated glucosinolates, flavonoids, phenolics, and terpenoids. Ground seed can also be extracted with solvents, such as hexane, or by newer "green" extraction technologies, such as supercritical carbon dioxide or high-pressure and temperature ethanol, to produce a powdered meal that contains less than 1% residual oil.

7.4.1 Carbohydrates

Carbohydrates found in camelina seed meal include mono-, di-, tri-, and tetra-saccharides, along with both oligo- and polysaccharides in the form of starch, pectin, and fiber of which a substantial part is composed of cellulose (Table 7.5 and [26]).

Table 7.5 Weight percent of carbohydrates, fiber, and phytic acid in solvent-defatted camelina seed meal

Sugar	Berhow	Zubr [26]
Glucose	0.02%	0.42%
Fructose	0.01%	0.04%
Sucrose	5.60%	5.50%
Raffinose	0.80%	0.64%
Stachyose	0.30%	0.36
Verbascose	0.08%	–
Total Soluble	8.60%	6.96%
Mucilage	–	6.70%
Starch (measured as glucose)	0.52%	1.21%
Pectin	–	0.96%
Acid-hydrolyzable uronic acid (pectin)	1.21%	–
Acid-hydrolyzable fructan	0.42%	–
Acid-hydrolyzable arabinose	4.61%	–
Acid-hydrolyzable galactose	4.24%	–
Acid-hydrolyzable glucose	5.96%	–
Acid-hydrolyzable xylose	1.68%	–
Insoluble sugars (total)	18.64%	–
Crude fiber	–	12.80%
Lignans[a]	–	7.40%
Acid insoluble residue	9.96%	–
Ash	0.47%	–
Inositol hexaphosphate[b]	–	2.25%
Inositol pentaphosphate[b]	–	0.10%

[a] Table 7.9
[b] [28]

The most interesting carbohydrate component is the mucilage that is formed after the addition of water, which forms a gel that can be isolated as a separate component that may be useful as a gum or tackifier [6, 26, 27]. Some of the soluble disaccharides and polysaccharides can contribute to caloric intake, while the insoluble fiber and phenolic lignin precursors have good effects on gastrointestinal processes and health. Camelina does not contain appreciable levels of beta-glucan [11]. A percentage of the digestible carbohydrates is bound to a variety of proteins and phytochemicals and may be nutritionally available or utilized by gut microflora. Camelina, like many other plant species, accumulates significant amounts of phytic acid, a polyphosphorylated inositol sugar [28–30]. This compound can decrease mineral and protein bioavailability; however, some protective effects have also been described.

7.4.2 Proteins

The proteins of camelina meals are the least characterized of the camelina seed components [10]. Unlike for soy, wheat, rice, and peanuts, there has been no careful characterization of the storage proteins of camelina seeds. The amino acid

Table 7.6 Weight percent of amino acids in camelina seeds [10]

Amino acid	% protein	% seeds
Alanine	4.61%	1.96%
Arginine	8.15%	3.46%
Aspartic acid	8.71%	3.70%
Cysteine	2.12%	0.90%
Glutamic acid	16.40%	6.97%
Glycine	5.44%	2.31%
Histidine[a]	2.60%	1.10%
Isoleucine[a]	3.96%	1.68%
Leucine[a]	6.63%	2.82%
Lysine[a]	4.95%	2.10%
Methionine[a]	1.72%	0.73%
Phenylalanine[a]	4.19%	1.78%
Proline	5.09%	2.16%
Serine	5.04%	2.14%
Threonine	4.25%	1.81%
Tryptophan[a]	1.15%	0.49%
Tyrosine	3.04%	1.29%
Valine[a]	5.42%	2.30%

[a] Dietary essential amino acid

composition has been examined [23], and a few studies have looked at trypsin inhibitor activity. In one study, the trypsin inhibitor activity was found to be between 16 and 21 units per mg on a dry weight basis [11], high enough to warrant some concerns. However, the activity could be alleviated by heat treatment, and sufficient variation exists to indicate that it could also be minimized through selective cultivar breeding. Camelina has at least 18 amino acids, of which nine are essential (Table 7.6 and [23]). No allergenic proteins or peptides have been detected in camelina.

7.4.3 Phytochemicals and Other Components

One of the more difficult aspects of plant chemical compositions to assess is the group of compounds aggregately termed "phytochemicals". Phytochemicals are generally defined as "secondary metabolites" or "natural products"—those compounds produced by individual plant species, not inherently required to reproduce and maintain living cells, unlike the primary metabolites, which enables the plant species to chemically mediate environmental stresses such as microbial infestation, herbivore feeding, water stress, light stress, etc. [31]. Phytochemicals are distinct and characteristic to each plant species, and may be produced at various times in a plant's life cycle and accumulate in specific organs or even cell types. As such, they can be present in fairly significant quantities or in very minor quantities. They have been classified by their biosynthetic pathways: the major classes are the terpenes (isoprenoids), the phenolics (phenylpropanoids and polyphenols), and the alkaloids;

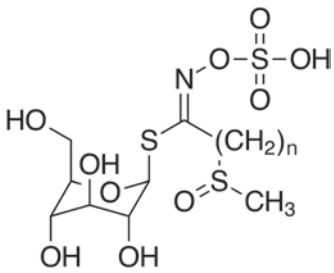


Fig. 7.1 Structure of glucosinolates found in camelina seeds. Glucoarabin (9-methylsulfinylnonyl-glucosinolate) $n=9$, glucocamelinin (10-methylsulfinyldecyl-glucosinolate) $n=10$, and 11-methylsulfinylundecyl-glucosinolate $n=11$

the minor ones which include sulfur-containing phytochemicals such as the glucosinolates, and other nitrogenous compounds such as the indoles and bioactive peptides. These minor phytochemical groups are often produced only by members of a few plant families. It is generally true that the types of phytochemicals found in a plant species/cultivar are always consistent, but the levels may vary considerably from cultivar to cultivar, from location to location, and from crop year to crop year. In many plant species—even the important crop species—not all of the phytochemicals have been completely characterized. This is true for camelina as well. Camelina seeds accumulate a suite of compounds presumably to facilitate germination and growth. These include terpenoids found in the oil fraction discussed above, lignans, tannins, flavonoids and other polyphenolics, and glucosinolates. Camelina seeds do not contain detectable levels of alkaloids, triterpenoid glycosides, or indoles.

7.4.3.1 Glucosinolates

Glucosinolates occur as secondary metabolites in many plants of the order Brassicales (especially in the Brassicaceae, as well as in members of the Capparidaceae and Caricaceae), with about 120 different glucosinolates known to occur naturally [32–34]. The plants contain the enzyme myrosinase, which in the presence of water liberates glucose. The remaining part of the molecule is quickly converted to either a thiocyanate, an isothiocyanate, or a nitrile; these are the active substances that serve as chemical defenses for the plant. Glucosinolates are well known for their toxic effects (mainly as goitrogenic agents) in both humans and animals at high doses. In contrast, at sub-toxic doses, their hydrolytic and metabolic products act as chemoprotective agents against chemically induced carcinogens by blocking the initiation of tumors in a variety of mammalian tissues. They exhibit their effect by inducing phase I and phase II enzymes, by inhibiting enzyme activation, modifying steroid hormone metabolism, and protecting against oxidative damages [32, 35–37].

Camelina accumulates significant levels of just three glucosinolates in its seeds: glucoarabin (9-methylsulfinylnonyl-glucosinolate), glucocamelinin (10-methylsulfinyldecyl-glucosinolate), and 11-methylsulfinylundecyl-glucosinolate (Fig. 7.1, Table 7.7) [38–40]. As with all phytochemicals, this accumulation is greatly

Table 7.7 Weight percent of glucosinolates in camelina seeds

	Berhow	Berhow	Shuster [40]
Glucosinolate	*Defatted*	*Whole*	*Whole*
Glucoarabin (GS9)	0.55%	0.47%	0.29%
Glucocamelinin (GS10)	1.35%	1.15%	0.80%
11-(Methylsulfinyl) undecyl-GS (GS11)	0.20%	0.17%	0.15%
Total	2.13%	1.79%	1.23%

affected by genotype and environmental growing conditions. The effect of the degradation products—the isothiocyanates, thiocyanates, and nitriles—from the camelina glucosinolates in diets and in agriculture has not been assessed, mainly due to a lack of purified standards for the necessary bioassays.

7.4.3.2 Flavonoids

Flavonoids and the (biosynthetically) related coumarins are ubiquitous in the plant kingdom, though most plant species accumulate significant levels of only a select few. No other class of phytochemicals has been credited with so many and such diverse functions in plant growth and survival as the flavonoids [41]. Not only are these compounds involved in defense against pathogens, herbivores, and other plants, but they also function in plant reproduction, mineral absorption, and a variety of symbiotic relationships with species as diverse as bacteria, insects, birds, and humans. Many flavonoids, along with benzoic acid derivatives discussed below, function as antioxidants, which allow these compounds to act as reducing agents, hydrogen donators, and singlet oxygen quenchers, an important role as natural antioxidants in foods [42].

In camelina plants, there are likely many flavonoids synthesized and utilized in leaves, stems, roots, and flowers, but in mature seeds only five are accumulated to detectable levels, the most significant of which are the glycosides of the flavonol quercetin [43–45]. Work in our lab has identified three forms of quercetin glycosides, resulting in a total concentration in the range of 10 mg/g (Fig. 7.2, Table 7.8). Other flavonoids that have been tentatively identified include an apigenin di-glycoside and a diosmetin di-glycoside, both found at relatively low levels. The total flavonoid amounts in a seed extract was reported to be 143 mg/g based on spectroscopic measurements, but these are likely inaccurate as many phenolics react with the chromogen reagent to produce absorbance at 640 nm [42]. Camelina has no reported levels of proanthocyanins or coumarins.

7.4.3.3 Mono- and Polyphenolics: Sinapine, Lignans, Tannins

The hydroxylated derivatives of benzoic and cinnamic acid are used by plants as the building blocks for flavonoids, coumarins, lignans, hydrolysable tannins, proanthocyanins, and a variety of other phenolic compounds. The phenolic compounds are

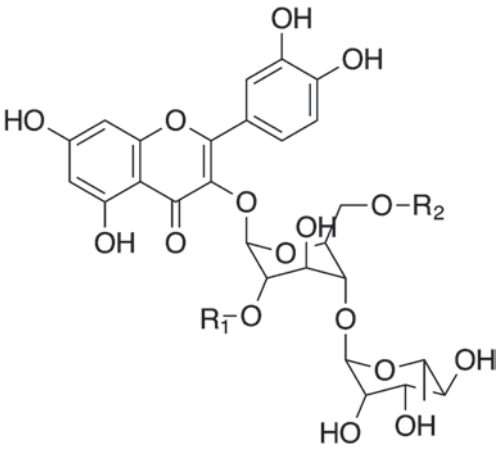


Fig. 7.2 Structure of quercetin glycosides in camelina seed meals. Rutin (quercetin-3-O-rutinoside) *R*1 = *R*2 = *H*; quercetin-2″-O-apiosyl-3-O-rutinoside *R*1 = apiose, *R*2 = *H*; quercetin-O-sinopyl-2″-O-apiosyl-3-O-rutinoside *R*1 = apiose, *R*2 (position not confirmed) = sinapic acid

the building blocks of many plant structural materials and they have a large array of biological activities including as antioxidants, as defense against pathogens and herbivores, as well as an array of dietary effects in humans and animals that consume them [30, 48].

Camelina seeds are reported to contain lignans, tannins, and some unbound phenolic acids (Table 7.9 and [28, 42, 45–48]). Camelina seeds contain sinapine, the choline ester of sinapic acid, at levels potentially as high as 60 mg/g [42]. Sinapine is present in many other Brassicaceae species and has several undesirable properties as a constituent in animal feeds. It is bitter tasting, thus rendering it less palatable to animals. It is possible that natural variability or breeding programs will lead to camelina cultivars with much lower levels of sinapine.

Table 7.8 Flavonoids and phenolics in whole camelina seeds

RT	Class	Name	Weight %
5	Phenolic	Chlorogenic acid derivative	0.07%
9	Phenolic	Sinapine	0.22%
12	Phenolic	Ellagic acid	0.11%
13	Phenolic	Sinapic acid	0.10%
16	Phenolic	Phenolic acid	0.06%
23.2	Flavonoid	Quercetin-2″-O-apiosyl-3-O-rutinoside	0.27%
23.4	Flavonoid	Flavone di-glycoside	0.11%
26	Flavonoid	Quercetin-3-O-rutinoside (rutin)	0.52%
27	Flavonoid	Kaempferol-O-rutinoside	0.07%
28.8	Flavonoid	Quercetin-O-sinopyl-2″-O-apiosyl-3-O-rutinoside	0.05%
29.2	Flavonoid	Isorhamnetin-O-rutinoside	0.08%
Total			1.61%

RT retention time in HPLC elution profile in minutes running a 250 × 4.6 C-18 reverse-phase column at 1 mL per min. The column was developed over 50 min with a liner gradient from 20% methanol and 80% 0.01M phosphoric acid to 100% methanol

Table 7.9 Sinapine, lignans, and tannins in whole camelina seeds [47, 48]

Compound	Weight percent
Sinapine[a]	0.50%
Protocatechuic acid	–
p-Hydroxybenzoic	–
Ellagic acid	–
(+)-Secoisolariciresinol	1.52%
(+)-Pinoresinol	0.98%
(+)-Syringaresinol	0.71%
(−)-Lariciresinol	2.18%
(−)-7-hydroxymatairesinol	0.04%
(+)-Medioresinol	0.65%
Cyclolariciresinol	0.12%
Secoisolariciresinol-sesquilignan	0.04%
Matairesinol	0.03%
Lariciresinol-sesquilignan	0.02%
Tannins[b]	0.20%
Total	6.58%

[a] [42] notes as high as 5%
[b] Tannins expressed as gallic acid equivalents

Table 7.10 Vitamins in camelina full fat seed meal [26]

Vitamin	Weight %
Thiamin (B1)	0.0019%
Riboflavin (B2)	0.0004%
Niacin (B3)	0.0194%
Panthothenic acid (B5)	0.0011%
Pyridoxine (B6)	0.0002%
Biotin (B7)	0.0001%
Folate (B9)	0.0003%

7.4.3.4 Vitamins

Camelina seeds contain detectable levels of several vitamins. Besides vitamin E in the oil fraction, the seeds contain several of the B vitamins (Table 7.10) [26] and are considered to be a good source of thiamin (B1), niacin (B3), and panthothenic acid (B5).

7.4.3.5 Minerals

Camelina has appreciable levels of several essential dietary minerals (Table 7.11) [26]. There is ancillary evidence that, like other Brassicaceae species, it is capable of sequestering heavy metals such as cadmium. This should be considered when growing camelina in areas that have high levels of toxic minerals in the soil.

Table 7.11 Minerals in camelina full fat seed meal [26]

Mineral	Weight %
Calcium	1.00 %
Magnesium	0.51 %
Sodium	0.06 %
Potassium	1.60 %
Chlorine	0.04 %
Phosphorus	1.40 %
Sulfur	0.24 %
Iron	0.0329 %
Copper	0.0099 %
Manganese	0.0040 %
Nickel	0.0002 %
Zinc	0.0069 %

7.4.4 Camelina Meal Uses

Due to the glucosinolate content, camelina meal has had only limited evaluation as a feed ingredient, but its use is slowly increasing. Recent research results have shown that adding defatted camelina meal to chicken [49], sheep [50], and cattle feeds [51, 52] has no discernable ill effects for meat, egg, and milk production animals. Press cake meals typically contain 10 % residual oil, which is high in omega-3 fatty acids and is somewhat enriched in phytosterols and tocopherols relative to expressed oil.

These observations, combined with the favorable amino acid composition of the protein, indicate that camelina meal is an excellent animal feed ingredient from a nutritional perspective. Current breeding work in the USA, Canada, and Finland, coupled with the analytical methodology discussed in this review, indicate that it is possible to reduce or eliminate some of the taste components of camelina that currently prevent higher percentages in feed blends. The unique character of the camelina glucosinolates, especially since they are similar in structure when converted to isothiocyanate form to the anticancer constituent sulforaphane identified in broccoli, makes camelina meal even more interesting as a functional food and as a source of nutraceuticals [33, 38].

7.5 Phytochemical Characterization Methods

Analytical methods and equipment are now well established and numerous chromatographic and spectrophotometric methods have been published for the analysis of all of the major classes of compounds found in camelina. In general, it can be concluded that (1) most spectrophotometric methods are inherently inaccurate; (2) most chromatographic methods are generally accurate and reproducible (as long as good calibration standards are available). Inaccuracies with the chromatographic

methods generally come from poor and inconsistent sample preparation methods which either do not allow for the maximum extraction of the components of interest, and/or enhances the loss of the compounds of interest through binding to clean-up and concentration methods or alteration/degradation by chemical and physical effects. The best general sample preparation method is to start with dry samples, grind them into as fine and uniformly-sized powder as possible with minimal addition of heat, extract with the maximum ratio of solvent to solid as possible, using heat or sonication to aid the process, then run the analysis on the samples with as little postextraction cleanup as possible.

For camelina, analysis should be performed on whole seeds, which can be used to compare to analytical results obtained on processed samples. Seeds can be ground with a variety of mills—for instance, coffee grinders. Because the seeds are small, sieving is not generally required.

For accurate measurement of the oil and seed meal components, they should be separated by extraction with a nonpolar solvent such as hexane. Care should be taken to prevent volatile loss if one is interested in headspace analysis [19]. Unrefined oil can be quantified after derivatization to fatty acid methyl esters using conventional GC-FID methodology [15]. Analysis of sterols, tocopherols, and terpenes is accomplished by HPLC after recovery of unsaponifiables [17, 24, 53]. The defatted seed meal may be extracted using a variety of polar solvents (water, methanol, DMSO, ethyl acetate, dichloromethane, etc.) to isolate polar constituents for further quantification using chromatographic methods such as HPLC. Some indirect spectrophotometric methods or digestion methods are needed to obtain values for starch, pectin, cellulose amounts [26], or for amino acid [23] and lignin composition [48]. Quantitative analysis of the water-soluble oligosaccharides from camelina meal is achieved by high-performance anion-exchange chromatography-pulsed amperometric detection [54–56]. The bound carbohydrates in the remaining sample can then be hydrolyzed with trifluroacetic acid and per-acetylated for subsequent GC analysis [57].

Free phenolics, flavonoids, and many other glycosylated phytochemicals can be measured by a variety of reverse phase HPLC methodologies as long as the proper absorption wavelength and standards are used [58, 59]. The emergence of accurate mass spectrometry as a benchtop detection system makes the identification of known chemical species even more straightforward. Glucosinolate analysis is more complicated. The original methodology was to analyze desulfonated forms of the glucosinolates [33, 40, 47], but if good ion-pairing agents are used, reverse phase chromatographic analysis can be carried out on intact glucosinolates extracted from the meal [33, 38, 60].

Currently there remain a few uncharacterized components of camelina seeds. Comprehensive evaluation of the seed proteins would essentially complete the compositional profile of camelina. For phytochemicals, we have used the latest accurate mass LC-MS analysis to quickly characterize the potential chemical formulas for a series of unknown compounds. We were able to tentatively identify several phenolics in the methanol extracts of defatted camelina seeds including

rutin (quercetin-3-O-rutinoside), and two derivatives of rutin—quercetin-2″-O-apiosyl-3-O-rutinoside and quercetin-O-sinopyl-2″-O-apiosyl-3-O-rutinoside. The identification of the latter two compounds was accomplished in a single CID/HCD mass fragmentation experiment in which interpretation of the daughter ions allowed for the identification of the aglycone and the various substitute fragments (Fig. 7.3). This result—coupled with DEPT NMR analysis of a partially purified compound and the observation that the isolated compound degraded to rutin—resulted in the identification of these phytochemicals in camelina.

The original M-H^- accurate mass ion of the peak at retention time 23.8 min was 741.18518 providing a chemical formula for the intact compound of $C_{32}H_{38}O_{20}$. The upper trace in Fig. 7.3 shows the UV absorbance at 280 nm, the second trace shows the SIM trace for the negative ion *m/z* 741, the third panel shows the identification of the daughter mass ions produced after collision-induced-fragmentation at 30% energy level on the original ion *m/z* 741. *M* = negative ion of the original compound, Api = loss of apiose mass, Rha = loss of rhamnose mass, Glu = loss of glucose mass. The aglycone of the compound is quercetin (M-H^- *m/z* 301), which also gives rise to an ion M-$2H^-$ (*m/z* 300) in the fragmentation. The ions resulting from the losses of apiose, rhamnose, and glucose were identified in the spectrum.

The attachment point of the apiose sugar was determined to be the 2″-position of the glucose moiety from the observation that fragmentation formed ions that were the result of the loss of two-thirds of the glucose (and the rhamnose attached to it), but the apiose moiety remained attached to one of those fragments (*m/z* 475) [61]. The identity of the unknown third 5-carbon sugar as apiose was determined by DEPT NMR on a partially purified isolate prepared from the methanol extract of camelina defatted seed meal.

Similarly, a peak with a later retention time of 27 min had a negative mass ion of 947.24225, which provided a chemical formula for the intact compound of $C_{43}H_{48}O_{24}$. It was identified by accurate mass fragmentation showing the loss of sinapic acid to form an ion *m/z* 741, which was further fragmented into ions *m/z* 609 and *m/z* 300, matching the fragmentation pattern found for quercetin-2″-O-apiosyl-3-O-rutinoside. This compound was tentatively identified as quercetin-O-sinopyl-2″-O-apiosyl-3-O-rutinoside.

The rapid advances in chromatography equipment have made the reliable and reproducible measurement of a wide range of plant chemical components possible. These accurate measurements on a limited scale can be coupled to more rapid nondestructive spectrophotometric analytical methods such as pulsed NMR and near infrared (NIR) spectroscopy, which will allow for the rapid and nondestructive analysis of thousands of samples for a wide range of physical and chemical composition parameters.

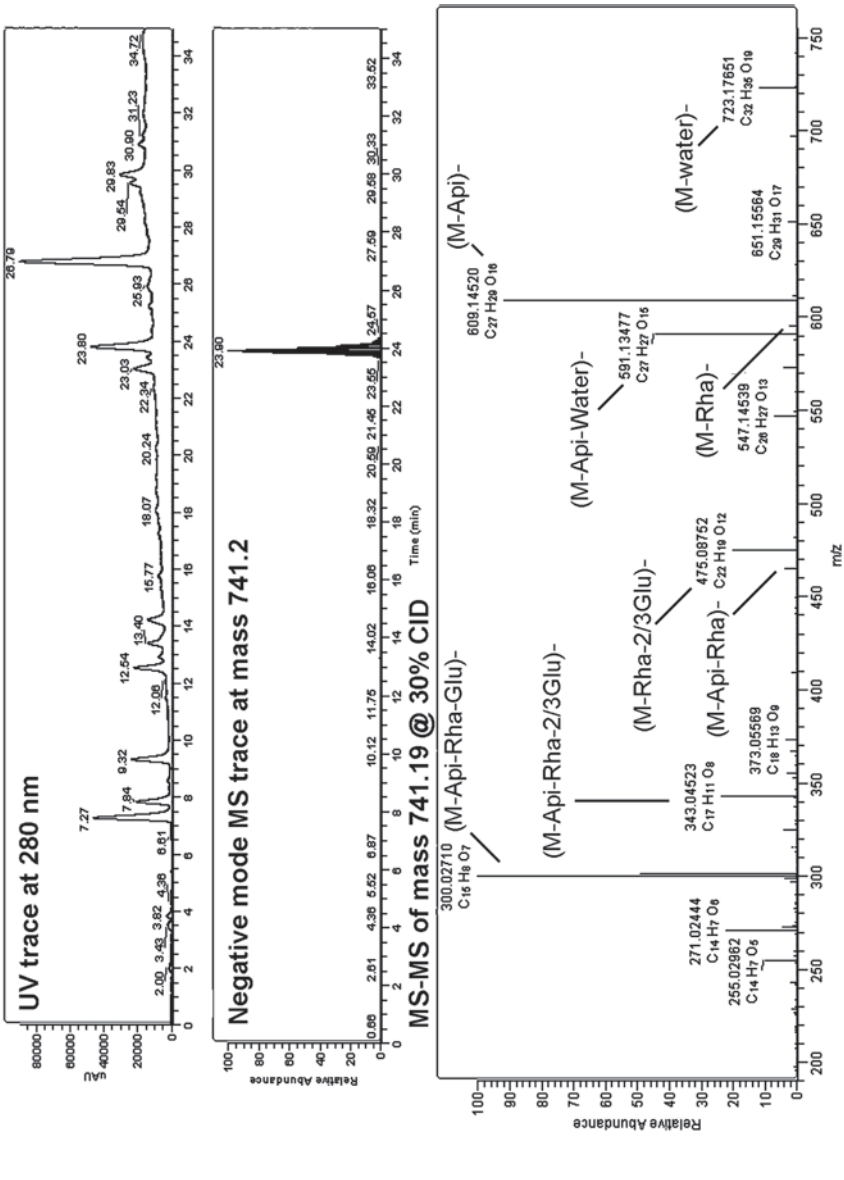


Fig. 7.3 Accurate mass MS–MS analysis used to characterize the unknown flavonol peak at retention time 23.8 min. The extract was run on a Thermo Scientific Accela UHPLC system and mass spectra were obtained on LTQ Orbitrap Discovery Mass Spectrometer (a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with HCD cell, using an Ion Max electrospray ionization (ESI) source

References

1. Gugel RK, Falk KC (2006) Agronomic and seed quality evaluation of Camelina sativa in western Canada. Can J Plant Sci 86:1047–1058
2. Zeman N (2007) Crazy for Camelina. Biodiesel Magazine, February 2007. http://biodieselmagazine.com/articles/1438/crazy-for-camelina
3. Jones G, Valamoti SM (2005) Lallemantia, an imported or introduced oil plant in Bronze Age northern Greece. Veg Hist Archaeobotany 14:571–577
4. Jones G, Valamoti SM (2006) Erratum: Lallemantia, an imported or introduced oil plant in Bronze Age northern Greece. Veg Hist Archaeobotany 16:71
5. Ehrensing DT, Guy SO (2008) Camelina, Oregon State University Extension Service EM 8953-E, January 2008: Corvallis http://wwwir.library.oregonstate.edu/xmlui/bitstream/handle/1957/20552/em8953-e.pdf
6. McVay KA, Lamb PF (2008) Camelina production in montana. Montana State University Extension Office, Bozeman. http://wwwmsuextension.org/publications.asp
7. Putnam DH, Budin JT, Field LA, Breene WM (1993) Camelina: a promising low-input oilseed. In: Janick J, Simon JE (ed) New Crops. Wiley, New York, pp 314–322
8. Moser BR (2010) Camelina (*Camelina sativa* L.) oil as a biofuels feedstock: golden opportunity or false hope? Lipid Technol 22:270–273
9. Vollmann J, Moritz T, Kargl C, Baumgartner S, Wagentristl H (2007) Agronomic evaluation of camelina genotypes selected for seed quality characteristics. Ind Crop Prod 26:270–277
10. Zubr J (2003). Qualitative variation of *Camelina sativa* seed from different locations. Ind Crop Prod 17:161–169
11. Budin JT, Breene WM, Putnam DH (1995) Some compositional properties of Camelina (Camelina sativa L. Crantz) Seeds and Oils. J Am Oil Chem Soc 72:309–315
12. Dubois V, Breton S, Linder M, Fanni J, Parmentier M (2007) Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. Eur J Lipid Sci Technol 109:710–732
13. Frohlich A, Rice B (2005) Evaluation of Camelina sativa oil as a feedstock for biodiesel production. Ind Crop Prod 21:25–31
14. Moser BR (2012) Biodiesel from alternative feedstocks: Camelina and field pennycress. Biofuels 3:193–209
15. Moser BR, Vaughn SF (2010) Evaluation of alkyl esters from Camelina sativa oil as biodiesel and as blend components in ultra low-sulfur diesel fuel. Bioresour Technol 101:646–653
16. Sampath A (2009) Chemical characterization of Camelina seed Oil. In: Food Science Rutgers, the State University of New Jersey, New Brunswick, p 193
17. Szterk A, Roszko M, Sosińska E, Derewiaka D, Lewicki PP (2010) Chemical composition and oxidative stability of selected plant oils. J Am Oil Chem Soc 87:637–645
18. Zubr J, Matthaus B (2002) Effects of growth conditions on fatty acids and tocopherols in Camelina sativa oil. Ind Crop Prod 15:155–162
19. Krist S, Stuebiger G, Bail S, Unterweger H (2006) Analysis of volatile compounds and triacylglycerol composition of fatty seed oil gained from flax and false flax. Eur J Lipid Sci Technol 108:48–60
20. Dubois V, Breton S, Linder M, Fanni J, Parmentier M (2007) Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. Eur J Lipid Sci Technol 109:710–732
21. Hutcheon C, Ditt RF, Beilstein M, Comai L, Schroeder J, Goldstein E, Shewmaker CK, Nguyen T, De RJ, Kiser J (2010) Polyploid genome of Camelina sativa revealed by isolation of fatty acid synthesis genes. BMC Plant Biol 10:233
22. Peiretti PG, Meineri G (2007) Fatty acids, chemical composition and organic matter digestibility of seeds and vegetative parts of false flax (Camelina sativa L.) after different lengths of growth. Anim Feed Sci Technol 133:341–350
23. Zubr J (2003) Dietary fatty acids and amino acids of Camelina sativa seeds. J Food Qual 26:451–462

24. Schwartz H, Ollilainen V, Piironen V, Lampi AM (2008) Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. J Food Compos Anal 21:152–161
25. Shukla VKS, Dutta PC, Artz WE (2002) Camelina oil and its unusual cholesterol content. JAOCS, J Am Oil Chem Soc 79:965–969
26. Zubr J (2010) Carbohydrates, vitamins, and minerals of Camelina sativa seed. Nutr Food Sci 40:523–531
27. Vaughn SF, Kenar JA, Felker FC, Berhow MA, Cermak SC, Evangelista RL, Fanta GF, Behle RW, Lee E (2013) Evaluation of alternatives to guar gum as tackifiers for hydromulch and as clumping agents for biodegradable cat litter. Ind Crop Prod 43:798–801
28. Matthaus B (2004) Antinutritional factors in different oilseeds usable as renewable resources compared with rapeseed. In: Muzquiz M et al (eds) Recent advances of research in antinutritional factors in legume seeds and oilseeds. Academic Publishers, Wageningen, pp 63–67
29. Matthaus B, Angelini L (2005) Anti-nutritive constituents in oilseed crops from Italy. Ind Crop Prod 21:89–99
30. Matthaus B, Zubr J (2000) Variability of specific components in Camelina sativa oilseed cakes. Ind Crop Prod 12:9–18
31. Finn R (2010) Nature's chemicals: the natural products that shaped our world. Oxford University Press, New York
32. Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56:5–51
33. Clarke DB (2010) Glucosinolates, structures and analysis in food. Anal Method 2:310–325
34. Daxenbichler ME, Spencer GF, Carlson DG, Rose GB, Brinker AM, Powell RG (1991) Glucosinolate composition of seeds from 297 Species of wild plants. Phytochemistry 30:2623–2638
35. Anilakumar KR, Khanum F, Bawa AS. (2006) Dietary role of glucosinolate derivatives: a review. J Food Sci Technol 43:8–17
36. Talalay P, Fahey JW (2001) Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 131:3027S–3033S
37. Vig AP, Rampal G, Thind TS, Arora S (2009) Bio-protective effects of glucosinolates—a review. Food Sci Technol 42:1561–1572
38. Berhow MA, Polat U, Glinski JA, Glensk M, Vaughn SF, Isbell T, Ayala-Diaz I, Marek L, Gardner C (2013) Optimized analysis and quantification of glucosinolates from Camelina sativa seeds by reverse-phase liquid chromatography. Ind Crop Prod 43:119–125
39. Lange R, Schumann W, Petrzika M, Busch H, Marquard R (1995) Glucosinolates in linseed dodder. Fett 97:146–152
40. Schuster A, Friedt W (1998) Glucosinolate content and composition as parameters of quality of Camelina seed. Ind Crop Prod 7:297–302
41. Andersen OM, Markham KR. (2006) Flavonoids: chemistry, biochemistry and applications. CRC Press, Taylor
42. Matthaus B (2002) Antioxidant activity of extracts obtained from residues of different oilseeds. J Agric Food Chem 50:3444–3452
43. Onyilagha J, Bala A, Hallett R, Gruber M, Soroka J, Westcott N (2003) Leaf flavonoids of the cruciferous species, Camelina sativa, Crambe spp., Thlaspi arvense, and several other genera of the family Brassicaceae. Biochemical Systematics and. Ecology 31:1309–1322
44. Onyilagha JC, Gruber MY, Hallett RH, Holowachuk J, Buckner A, Soroka JJ (2012) Constitutive flavonoids deter flea beetle insect feeding in Camelina sativa L. Biochem Syst Ecol 42:128–133
45. Terpinc P, Polak T, Makuc D, Ulrih NP, Abramovic H (2003) The occurrence and characterization of phenolic compounds in Camelina sativa seed, cake and oil. Biochem Syst Ecol 31:1309–1322
46. Matthaus B, Zubr J (2000) Bioactive compounds in oil-cakes of Camelina sativa (L.) Crantz. Agro-Food-Ind Hi-Tech 11:20–24
47. Matthaus B, Zubr J (2000) Variability of specific components in Camelina sativa oilseed cakes. Ind Crop Prod 12:9–18

48. Smeds AI, Eklund PC, Willfor SM (2012) Content, composition, and stereochemical characterisation of lignans in berries and seeds. Food Chem 134:1991–1998
49. Kakani R, Fowler J, Haq AU, Murphy EJ, Rosenburger TA, Berhow MA, Bailey CA (2012) Camelina meal increases egg n-3 fatty acid content without altering egg quality or production in laying hens. Lipids 47:519–526
50. Mierlita D, Daraban S, Lup F, Chereji A (2011) The effect of grazing management and camelina seed supplementation in the diet on milk performance and milk fatty acid composition of dairy ewes. J Food Agric Environ 9:368–373
51. Halmemies-Beauchet-Filleau A, Kokkonen T, Lampi AM, Toivonen V, Shingfield KJ, Vanhatalo A (2011) Effect of plant oils and camelina expeller on milk fatty acid composition in lactating cows fed diets based on red clover silage. J Dairy Sci 94:4413–4430
52. Moloney AP, Woods VB, Crowley JG (1998) A note on the nutritive value of camelina meal for beef cattle. Irish J Agric Food Res 37:243–247
53. Gruszka J, Kruk J (2007) RP-LC for determination of plastochromanol, tocotrienols and tocopherols in plant oils. Chromatographia 66:909–913
54. Giannoccaro E, Wang YJ, Chen P (2006) Effects of solvent, temperature, time, solvent-to-sample ratio, sample size, and defatting on the extraction of soluble sugars in soybean. J Food Sci 71:C59–C64
55. Giannoccaro E, Wang YJ, Chen P (2008) Comparison of two HPLC systems and an enzymatic method for quantification of soybean sugars. Food Chem 106:324–330
56. Hou A, Chen P, Shi A, Zhang B, Wang Y-J (2009) Sugar variation in soybean seed assessed with a rapid extraction and quantification method. Int J Agron 2009:1–8
57. Price NPJ (2004) Acylic sugar derivatives for GC/MS analysis of 13C-enrichment during carbohydrate metabolism. Anal Chem 76:6566–6574
58. Berhow MA (2002) Modern analytical techniques for flavonoid determination. In: Buslig BS, Manthey JA (eds) Flavonoids in the Living Cell. Kluwer Academic/Plenum Publishers, New York, pp 61–76
59. Berhow MA, Kong S-B, Duval SM (2006) Complete quantification of group A and group B Saponins in Soybeans. J Agric Food Chem 54:2035–2044
60. Betz JM, Fox WD (1994) High-performance liquid chromatographic determination of glucosinolates in Brassica vegetables. In: Huang M-T et al (eds) Food Chemicals for Cancer Prevention I: Fruits and Vegetables, ACS Symposia series 546. ACS publications, Washington, DC, pp 181–195
61. Vulkas V, Guttman A (2010) Structural characterization of Flavonoid Glycosides by multistage mass spectrometry. Mass Spectrom Rev 29:1–16

Chapter 8
Analysis of β-Methylamino-Alanine in Environmental and Food Samples

W. Broc Glover and Susan J. Murch

Abstract The naturally occurring, non-protein amino acid N-β-methylamino-L-alanine (BMAA) is produced by cyanobacteria in many different parts of the world. Beginning with field studies performed in Guam in the early 1960s, evidence has correlated chronic exposure to low levels of BMAA in the diet to an increased risk of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia. In animal model systems and in vitro bioassays, BMAA has been shown to damage neurons and induce neurodegeneration. However, inconsistency in analytical methods has led to conflicting data and controversial interpretations, and accurate analytical methods for the detection and quantification of BMAA are required. This review provides an overview of analytical methods used for BMAA analysis, including sample preparation and clean-up protocols, derivatization, chromatography, and mass spectrometry. A number of factors that should be carefully considered are identified, including solvent interactions, ion suppression, and the formation of metal adducts and complexes. Advancements in our understandings of these areas will lead to better methods for identification and quantification of BMAA in biological and environmental samples, and will help to resolve the ongoing controversy about the potential role(s) of BMAA in human health.

Keywords β-Methylamino-L-alanine (BMAA) · 2,4-Diaminobutanoic acid (DAB) · N-(2-Aminoethy)glycine (AEG) · β-Amino-N-methylalanine (BAMA) · Natural neurotoxin

8.1 Introduction

In 1952 and 1953, reports appeared in the medical literature describing an epidemic of amyotrophic lateral sclerosis (ALS) on the island of Guam [1–3]. The earliest documented case of the disease that could be confirmed by a death certificate

S. J. Murch (✉) · W. B. Glover
Department of Chemistry, University of British Columbia,
Okanagan Campus, 3333 University Way, Kelowna, BC V1V 1V7, Canada
e-mail: susan.murch@ubc.ca

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_8

occurred in 1904 but the indigenous Chamorro people had a long memory of the disease that they called “lytico” [4]. A “house-to-house” survey of three villages in 1953 concluded that the prevalence of ALS among the Chamorro on Guam and Rota was about 420 per 100,000 in the population, as compared to ca. 4/100,000 in North America and Europe [4, 5]. The disease accounted for 8–10 % of the adult Chamorro deaths per year, and the highest incidence was found among the most traditional families [5]. Further studies diagnosed a second clinical manifestation of the disease that was identified as “parkinsonism dementia” (PD), and the disease became broadly known as amyotrophic lateral sclerosis/parkinsonism dementia complex ALS/PDC [6]. Understanding the causes of ALS/PDC in Guam could provide the keys to understanding progressive neurodegenerative diseases elsewhere.

In 1960, the indigenous Chamorro population of Guam was 34,762, including approximately 40,000 American military personnel and related staff or family, 6,000 Filipino contract workers and 600 Caroline Islanders [6]. The disease incidence was limited to the Chamorro in Guam and Chamorro who had moved to California, suggesting a genetic or hereditary basis for the disease (reviewed in [6]). From 1962 to 1964, two cases of the disease were reported among the non-Chamorro population [6–8]. These cases, along with detailed statistical analysis of the Chamorro population, lead to the conclusion that the risk of the disease is clustered among families but not genetically determined [7, 9, 10]. A non-Chamorro living in the Chamorro community had an equal chance of becoming ill [6]. Likewise, the possibilities of viral infections, transmissible agents from animals, micronutrient deficiencies, and calcium deficiency were all eliminated, leading to the conclusion that the disease was most likely the result of “long-term exposure to the environment” (reviewed in [6, 11]). Over more than 40 years of study, the only factor that was positively correlated with the disease incidence was a preference for traditional Chamorro foods [11].

8.1.1 Discovery and Chemistry of β-Methylamino-Alanine (BMAA)

The ethnobotanist Marjory Whiting conducted extensive field studies in Guam to document the Chamorro culture and to determine whether traditional foods might be implicated in ALS/PDC [12]. Whiting identified flour made from the seeds of the indigenous cycad (*Cycas micronesica* Hill; Fig. 8.1a) as a potentially toxic food source [12], tested the flour on rats [13], and submitted samples of cycad seeds for analysis [12]. An extract of these Cycad seeds was neurotoxic to chicks and mice [14, 15], and found to contain relatively large concentrations of *β*–methylamino-alanine (BMAA, synonymous with *α*–amino-*β*-methylaminopropionic acid; Fig. 8.2) [14]. Throughout the 1970s and 1980s, the potential neurotoxicity of BMAA was extensively investigated in many different model systems and, most significantly, BMAA was found to be neurotoxic in primates when administered at high doses [16]. However, Duncan et al. (1992) argued that the traditional process of washing the cycad seeds in making the flour removed a significant proportion of the BMAA and that

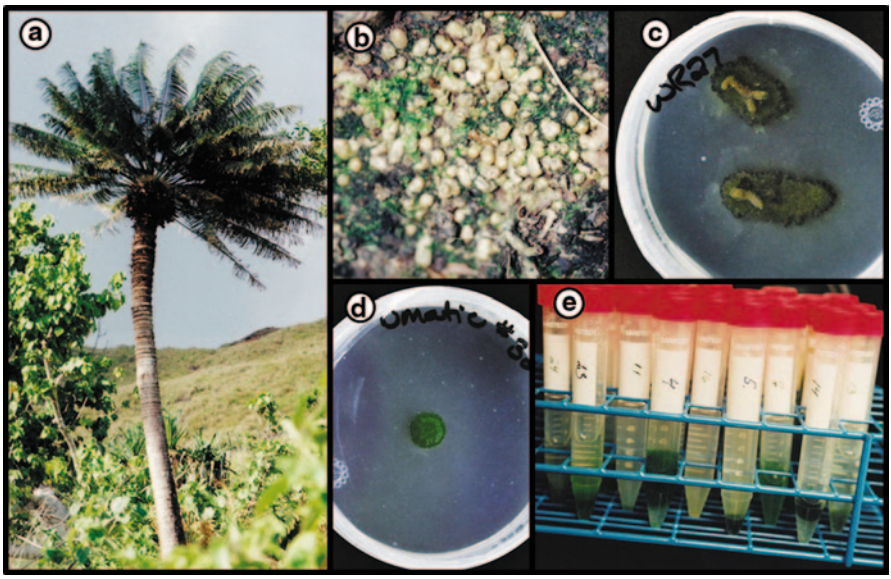
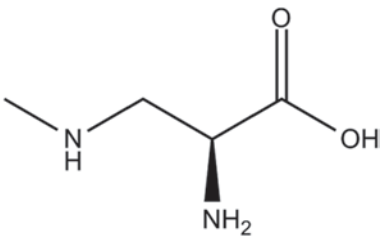


Fig. 8.1 Cyanobacteria isolated from roots of *Cycas micronesica* Hill on Guam. **a** *Cycas micronesica*. **b** Coralloid roots of *C. micronesica* with cyanobacteria infections. **c** Cyanobacteria were isolated from surface sterilized roots on standard BG11 media. **d** Sequential subcultures of cyanobacterial isolates produced axenic cultures on BG11 media. **e** A collection of cyanobacteria cultures from cycads at different locations on the island of Guam

Fig. 8.2 *β*–Methylamino-L-alanine (syn. *α*–amino-*β*-methylaminopropionic acid)



the remaining dose was not sufficient to cause the disease [17, 18] or that disease might be caused by zinc leaching from the wash buckets [19]. Research into the role of BMAA in progressive neurodegeneration was effectively suspended following these observations.

8.1.2 The BMAA Hypothesis

In 2002, Cox and Sacks proposed a hypothesis that re-invigorated research into the neurotoxicity of BMAA [20]. They hypothesized that BMAA could accumulate through different trophic levels of the ecosystem, resulting in different routes of

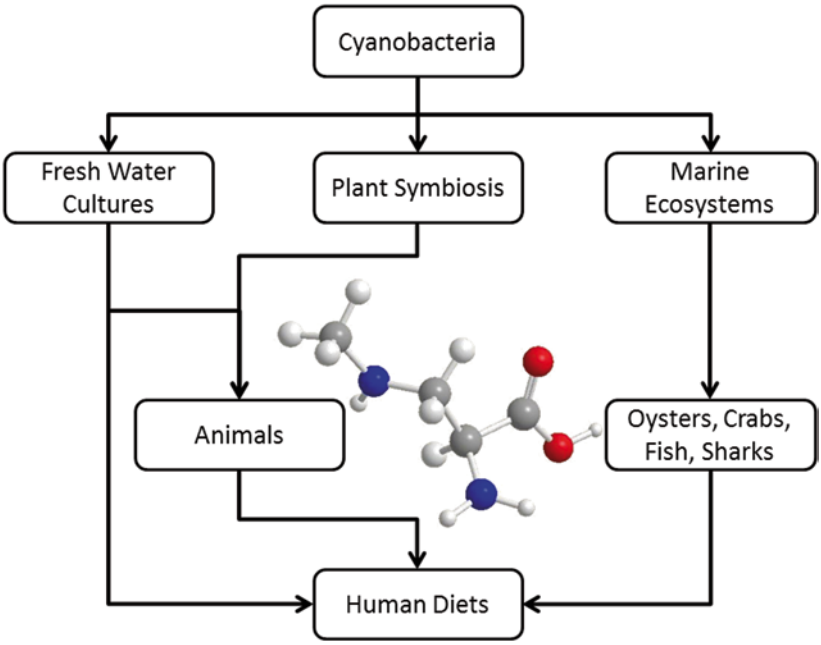


Fig. 8.3 The hypothesis that BMAA accumulates through trophic levels and can be consumed from many different parallel sources in the diet

exposure and biomagnification of the compound that would have increased the exposure of the Chamorro people. Research in Guam demonstrated that BMAA could be accumulated through the traditional Chamorro food web from cycads to animals including flying foxes, deer, and wild pigs [21–25]. In addition, BMAA was identified and quantified in brain autopsy samples of both Chamorro patients who died of ALS/PDC and Canadian Alzheimer's patients [26].

Roots of cycad trees form a symbiosis with cyanobacteria that induce the roots to grow upward through the soil surface (Fig. 8.1b). Cyanobacteria were isolated from surface sterilized roots collected at seven different sites in Guam (Fig. 8.1c) and established as axenic cultures (Fig. 8.1d). Analysis of these Guam cyanobacteria and international cyanobacteria collections (Fig. 8.1e) demonstrated that BMAA is produced by a diverse variety of symbiotic and free-living cyanobacteria including *Nostoc*, *Nodularia*, and *Trichodesmium* species [22, 27].

Together, these data suggest that BMAA is produced by cyanobacteria and accumulates in plants and animals that consume the plants, including humans who are exposed to unknown doses from multiple sources (Fig. 8.3). BMAA accumulation from cyanobacteria through food webs has been shown in blue crab, shrimp, and oysters in South Florida [28], mussels, oysters, and bottom dwelling fish of the Baltic [29] and traditional Chinese foods such as noodles [30] and shark fins [31] (Fig. 8.3). Also, BMAA has been identified and quantified in brain tissues of ALS and AD patients in Miami, Florida [32].

8.1.3 The Importance of Methods for Analysis of BMAA

Evaluating the BMAA hypothesis requires accurate analytical methods for detection, identification, and quantification of BMAA [33, 34]. Montine et al. (2005) reported that they were unable to detect BMAA in autopsy tissues of Chamorro ALS/PDC patients [35]. Several groups were unable to detect or quantify BMAA in cyanobacteria isolates [36–38]. To date, more than a dozen research groups have published 51 reports, with 42 confirming detection of BMAA and nine publications reporting a failure to detect BMAA (Table 8.1). Duncan (2012) stated "the role of BMAA in neurodegenerative disorders is fiercely debated. In large part, the controversy arises because of conflicting findings relating to the analytical work underpinning this complex web of observation and chemical analysis" (p. 804) [34]. The objective of this review is to highlight some of the most important issues, recent advancements and ongoing considerations in methods for analysis of BMAA in complex biological, environmental, and food samples.

8.2 Methods for Analysis of BMAA

The majority of analytical methods for detection and quantification of BMAA have used standard amino acid analysis techniques modified for low concentrations in complex samples. The early approaches used GC-MS [17, 18] or 9-fluorenylmethyl chloroformate (FMOC) derivatization followed by HPLC separation and fluorescence detection [39]. Our work, beginning in 2003, used a similar approach with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization prior to reverse phase chromatographic separation and fluorescence detection [21–23, 26]. The BMAA peak assignment was confirmed by LC-MS using an orthogonal method [22, 23, 26]. BMAA has also been successfully quantified in cyanobacterial isolates with a separate derivatization technique using propyl chloroformate derivatization (EZ:faast) and MS detection of the derivatized compound [40, 41]. Positive detection of BMAA was found in the majority of methods that employed a deriviatization reaction while the majority of methods failing to quantify BMAA attempted to detect underivatized BMAA by either MS or MS/MS. Additionally, the chromatographic separation seems to be important as many of the methods that failed to detect BMAA in biological samples attempted separation by hydrophilic interaction liquid chromatography (HILIC) rather than reverse phase LC-MS (Table 8.1; [36–38, 42, 43]. Recently, a study of the analysis of underivatized BMAA standards by MS found that the molecule is highly reactive and readily forms metal ion adducts and complexes in solution that complicate the analysis [44] (Table 8.2). No study has been done that compared the different approaches of different labs on a common set of samples or extracts.

Table 8.1 Methods of detection and quantification of BMAA in biological samples since 2003

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2003	[22]	Yes	RPLC	Waters SymetryShield RP-18	Acetonitrile/water	AQC	MS, UV	Agilent SL Single Quad, DAD
2003	[22]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2003	[21]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector
2004	[23]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	MS	Agilent SL Single Quad, DAD
2004	[23]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2004	[23]	Yes	RPLC	Waters symmetry column	Acetonitrile/water	AQC	MS, DAD	Agilent SL Single Quad, DAD
2004	[26]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Unspecified

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2005	[27]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	MS	Agilent SL Single Quad, DAD
2005	[27]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2006	[24]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	MS	Agilent SL Single Quad, DAD
2006	[24]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm×3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2007	[57]	Yes	Ion-exchange	Hitachi reaction column, Li-form resin and ion exchange ammonia filter column	Proprietary eluents (Hitachi)	Ninhydrin Derivatization (Post-Column)	FD	Hitachi Amino Acid Analyzer
2007	[57]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	0.1% formic acid in water/0.1% formic acid in acetonitrile	AQC	MS/MS	Thermo TSQ Quantum Discovery Max

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2007	[57]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	Waters AccQ-Tag Ulta Eluents A&B	AQC	MS	Waters EMD 1000 Single Quad
2007	[57]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 52% acetonitrile	AQC	FD	Waters 2475 Fluorescence Detector
2007	[57]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	Waters AccQ-Tag Ulta Eluents A&B	AQC	UV	Acquity UV Detector
2008	[40]	Yes	GC	ZB-AAA 10 m × 0.25 mm AAA GC column	Helium gas	ECF	EI-MS	Thermofinnigan Trace MS Plus
2008	[58]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	0.1% formic acid in water/0.1% formic acid in acetonitrile	AQC	MS	Waters EMD 1000 Single Quad
2008	[58]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	Waters AccQ-Tag Ulta Eluents A& B	AQC	UV	Acquity UV Detector
2008	[58]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector
2008	[58]	Yes	RPLC	Thermo Hypersil GOLD 100 mm × 2.1 mm, 3 μm column	0.1% formic acid in water/0.1% formic acid in acetonitrile	AQC	MS/MS	Thermo TSQ Quantum Discovery Max

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2008	[59]	Yes	RPLC	Thermo Hypersil GOLD 100 mm × 2.1 mm, 3 μm column	0.1 % formic acid in water/0.1 % formic acid in acetonitrile	AQC	MS/MS	Thermo TSQ Quantum Discovery Max
2008	[59]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60 % acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2009	[62]	Yes	RPLC	Thermo Hypersil GOLD 100 × 2.1 mm, 3 μm	0.1 % formic acid in water/0.1 % formic acid in acetonitrile	AQC	MS/MS	Thermo TSQ Quantum Discovery Max
2009	[43]	Yes	HILIC	SeQuant ZIC-HILIC, 5 μm, 150 × 2.1 mm	Acetonitrile/formic acid	None	MS/MS	Agilent G6410A QQQ
2009	[60]	Yes	RPLC	Waters Spherisorb 5 μm ODS2 (4.6 × 250 mm) × 2	Acetonitrile/formic acid	No	MS	Thermo LCQ DecaXP Plus LC/MSn equipped with APCI interface
2009	[61]	Yes	RPLC	Two serially coupled 3 mm i.d. Chromolith Performance RP-18e	20 mM sodium acetate, pH 5.5 and 50/50 acetonitrile/water	AQC	FD	Hitachi LaChrom fluorescence detector L-7485
2009	[30]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	0.1 % formic acid in water/0.1 % formic acid in acetonitrile	AQC	MS	Waters EMD 1000 Single Quad

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2009	[62]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2009	[32]	Yes	RPLC	Thermo Hypersil GOLD 100 × 2.1 mm, 3 μm	0.1% formic acid in water/0.1% formic acid in acetonitrile	AQC	MS/MS	Thermo TSQ Quantum Discovery Max
2009	[32]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60% acetonitrile	AQC	FD	Waters 2487 Dual-l Fluorescence Detector, Waters 2488 UV detector
2009	[63]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	0.1% formic acid in water/0.1% formic acid in acetonitrile	AQC	MS/MS	Thermo Finnigan Triple Quadrupole MS (model unspecified)
2010	[64]	Yes	RPLC	Waters C18 AccQ-Tag Ultra 2.1 × 100 mm column	Acetonitrile and 0.05% TFA; 70/30 (v/v), isocratic flow	AQC	MS/MS	Finnigan TSQ Quantum Ultra AM
2010	[65]	Yes	RPLC	Thermo Hypersil GOLD 100 mm × 2.1 mm, 3 μm column	Acetonitrile/formic acid	AQC	MS/MS	AB API 2000 Triple Quadrupole
2010	[29]	Yes	RPLC	Thermo Hypersil GOLD 100 mm × 2.1 mm, 3 μm column	Acetonitrile/formic acid	AQC	MS/MS	AB API 2000 Triple Quadrupole

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2010	[28]	Yes	RPLC	Waters Nova-Pak C18 column, 3.9 mm × 300 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.7 and 57 % (v/v) acetonitrile in water	AQC	FD	Waters 2475 Fluorescence Detector
2010	[28]	Yes	RPLC	Thermo Hypersil GOLD 100 × 2.1 mm, 3 μm	Acetonitrile/formic acid	AQC	MS/MS	Thermo TSQ Quantum Discovery Max
2011	[51]	Yes	RPLC	Phenomenex AAA-MS 250 2.0 mm, 4 μm	10 mM ammonium formate in methanol	Propylchrlorformate (EZ:faast)	MS/MS	Thermo Scientific Finnigan TSQ Quantum UltraAM
2011	[51]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60 % acetonitrile	AQC	MS	Waters EMD 1000 Single Quad
2011	[51]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 60 % acetonitrile	AQC	FD	Waters 2475 Multi Wavelengtgh Fluorescence Detector,
2011	[41]	Yes	RPLC	Phenomenex AAA-MS 250 2.0 mm, 4 μm	10 mM ammonium formate in water and 10 mM ammonium formate in methanol	PCF	MS	Shimadzu 2010EV

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2011	[48]	Yes	RPLC	Phenomenex AAA-MS 250 2.0 mm, 4 μm	10 mM ammonium formate in water and 10 mM ammonium formate in methanol	PCF	MS/MS	Thermo TSQ Quantum Discovery Max
2011	[48]	Yes	RPLC	Phenomenex AAA-MS 250 2.0 mm, 4 μm	10 mM ammonium formate in methanol	Propylchrlorformate (EZ:faast)	MS/MS	Thermo Scientific Finnigan TSQ Quantum UltraAM
2012	[55]	Yes	RPLC	Waters Nova-Pak C18 3.96 × 300 mm, 4 μm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 52 % (v/v) acetonitrile in water	AQC	FD	Agilent 1100 LC- FD
2012	[31]	Yes	RPLC	Waters Nova-Pak C18 column, 3.9 mm × 300 mm	140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 and 52 % (v/v) acetonitrile in water	AQC	FD	Waters 2475 Fluorescence Detector
2012	[31]	Yes	RPLC	Waters AccQTag Ultra column 2.1 × 100 mm	0.1 % formic acid in water/0.1 % formic acid in acetonitrile	AQC	MS/MS	Thermo Scientific Finnigan TSQ Quantum UltraAM
2012	[67]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine and 65 % methanol in water	AQC	FD	Merck Hitachi L-7480 Multi l-Fluorescence Detector

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2012	[53]	Yes	RPLC	Agilent Bonus RP Rapid Resolution High Throughput, 100 × 2.1 mm, 1.8 µm	Acetonitrile/formic acid	AQC	MS/MS	Thermo TSQ Vantage triple quad
2012	[66]	Yes	RPLC	Waters Nova-Pak C18 column, 300 mm × 3.9 mm	140 mM sodium acetate, 5.6 mM triethylamine and 65% methanol in water	AQC	FLD	Merck Hitachi L-7480 multi l-fluorescence detector
2012	[55]	Yes	RPLC	Agilent Zorbax Eclipse AAA 4.6 × 75 mm, 3.5 µm	Acetonitrile/formic acid	AQC	MS/MS	Agilent 6401A QQQ
2012	[44]	Yes	RPLC	Waters C18 AccQ-Tag Ultra 2.1 × 100 mm column	Acetonitrile/formic acid	AQC	MS	Waters LCT Premier XE TOF
2012	[68]	Yes	RPLC	Waters C18 AccQ-Tag Ultra 2.1 × 100 mm column	Acetonitrile and 0.05% TFA; 70/30 (v/v), isocratic flow	AQC	MS/MS	Finnigan TSQ Quantum Ultra AM
2005	[35]	No	RPLC	Unspecified	Unspecified	FMOC	FD	Unspecified
2008	[46]	No	HILIC	Tosoh TSK-gel Amide- 80 column (250 × 2.0 mm, 5 µm)	Acetonitrile/water	None	MS	Shimadzu LCMS 2010A
2008	[36]	No	HILIC	SeQuant ZIC-HILIC, 5 µm, 50 × 2.1 mm or 150 × 2.1 mm	Acetonitrile and 60 mM formic acid	None	MS/MS	Micromass Quattro Ultima or API 4000 QTRAP

Table 8.1 (continued)

Year	Reference	BMAA detected?	Chromatography method	Column ID	Elution buffer(s)	Derivative	Detection method	Detector
2009	[42]	No	GC×GC	60 m × 0.25 mm i.d. × 0.25 µm Restek RTX- 5MS	Helium gas	ECF	MS	HP 5971A GC/MS
2010	[37]	No	HILIC	Phenomenex Luna C18(2), 5 mm, 250 × 4.60 mm	2 mM ammonium formate (pH 3) in (A) water and (B) acetonitrile/water (90/10; v/v)	None	MS/MS	AB API 365 tandem mass spectrometer
2010	[64]	No	HILIC	SeQuant ZIC-HILIC, 5 µm, 150 × 2.1 mm	Acetonitrile and 60 mM FA; 60/40 (v/v), isocratic flow	None	MS/MS	Thermo Finnigan HPLC, Finnigan LCQ Deca XP Plus
2010	[65]	No	HILIC	Tosoh TSK-gel Amide- 80 column (250 × 2.0 mm, 5 µm)	Acetonitrile and 0.05% TFA; 70/30 (v/v), isocratic flow	None	MS/MS	Micromass Quattro Ultima
2012	[55]	No	HILIC	SeQuant ZIC-HILIC, 5 µm, 150 × 2.1 mm	Acetonitrile/formic acid	None	MS/MS	Agilent 6401A QQQ
2012	[38]	No	HILIC	Tosoh TSK-gel Amide- 80, 250 × 2.0 mm, 5 µm	Acetonitrile/formic acid	None	MS/MS	API 4000Qtrap MS

Table 8.2 Predicted adducts of BMAA in mass spectrometry analysis with electrospray ionization in positive mode [69]

Ion name	Charge	Ion mass	m/z
M+3H	3+	M/3+1.0073	40.3653
M+2H+Na	3+	M/3+8.3346	47.6927
M+H+2Na	3+	M/3+15.7662	55.1243
M+3Na	3+	M/3+22.9892	62.3473
M+2H	2+	M/2+1.0073	60.0444
M+H+NH4	2+	M/2+9.5201	68.5577
M+H+Na	2+	M/2+11.9982	71.0353
M+H+K	2+	M/2+19.9852	79.0223
M+ACN+2H	2+	M/2+21.5206	80.5577
M+2Na	2+	M/2+22.9892	82.0263
M+2ACN+2H	2+	M/2+42.0339	101.0709
M+3ACN+2H	2+	M/2+62.5471	121.5842
M+H	1+	M+1.0073	119.0815
M+NH4	1+	M+18.0339	136.1080
M+Na	1+	M+22.9892	141.0634
M+CH3OH+H	1+	M+33.0335	151.1077
M+K	1+	M+38.9632	157.0374
M+ACN+H	1+	M+42.0338	160.1080
M+2Na-H	1+	M+44.9712	163.0454
M+IsoProp+H	1+	M+61.0653	179.1395
M+ACN+Na	1+	M+64.0158	182.0900
M+2K+H	1+	M+76.9190	194.9932
M+DMSO+H	1+	M+79.0212	197.0954
M+2ACN+H	1+	M+83.0604	201.1346
M+IsoProp+Na+H	1+	M+84.0551	202.1293
2M+H	1+	2M+1.0073	237.1557
2M+NH4	1+	2M+18.0338	254.1822
2M+Na	1+	2M+22.9892	259.1376
2M+3H2O+2H	2+	2M+28.0231	264.1715
2M+K	1+	2M+38.9632	275.1116
2M+ACN+H	1+	2M+42.0338	278.1822
2M+ACN+Na	1+	2M+64.0158	300.1642

8.2.1 Sample Preparation

Preparation of the samples and extraction of BMAA from the sample matrix is the critical first step to ensure accurate analysis of BMAA. Many published methods begin with precipitation of the protein from samples using either 0.1 N trichloroacetic acid [22, 23, 26], water [45] and aqueous ethanol (50–80%)/dilute HCl [14, 36, 39, 46]. Other researchers subjected samples to freeze/thaw treatments in 80% methanol followed by ultrasonication [47]. The complete precipitation of proteins is essential since hydrolysis of the protein pellet with 6 N HCl at 110 °C increased the detectable concentrations of BMAA in cycad flour samples from Guam and could account for differences in the reported values of nearly six orders of magnitude [23]. Additional

considerations include the need to remove lipids that, if present in sample matrices, can interfere with MS detection. A liquid–liquid solvent partition using chloroform has been used by some researchers to remove lipids following acid hydrolysis [47].

8.2.2 Solid Phase Extraction

Solid phase extraction has been used in six reports of BMAA analysis with three of the manuscripts providing detailed procedures. Kubo et al. (2008) reported that BMAA, a highly polar molecule, is not effectively retained on C18 (Waters Sep-pak) or a lipophilic polymer (Waters Oasis HLB), but a polymeric cation exchange sorbent (Waters Oasis MCX), which contains a γ-lactam moiety to aid in the retention of polar analytes, readily retained BMAA [46]. Jiang et al. (2013) used an Isolute HCX SPE column for sample clean-up, added D_3-BMAA as an internal standard and reported a recovery of the D_3-BMAA of 63.3 % [47]. Downing et al. (2011) reported isolation and concentration of BMAA from drinking water using a polymeric sorbent containing a phenylsulphonic acid moiety (Phenomenex Strata X-C) [48]. Recovery of BMAA with this method was shown to be near 100 % for analyte spiked into distilled water, but much lower recoveries of 40–60 % were measured for other sample matrices. The authors propose that competition for the cation exchange sites between basic amino acids may reduce recovery [48]. Li et al. (2012) compared the relative recoveries of the Strata X-C and Oasis MCX systems [38]. Slightly improved recoveries (2–6 %) were reported for complex cyanobacterial samples with the Oasis cartridge despite using a much lower sorbent load (200 mg vs. 60 mg, respectively) [38]. Combined, these results suggest that SPE may be a highly effective cleanup and concentration procedure for BMAA analysis, but the type of cartridge must be carefully chosen and tested for individual matrices. As recovery is highly variable between different sorbents [38], it is important to include an internal standard during SPE clean-up steps to account for losses due to incomplete retention. Likewise, it is important that proper method development be performed when designing SPE methods so that BMAA is simultaneously not lost during wash steps, but also completely removed during the elution step. Without this, it is likely that false negatives could be obtained during the analysis of BMAA.

8.2.3 Derivatization

Derivatization facilitates detection of amino acids in low concentrations in complex sample matrices, improves chromatographic separation, improves the selectivity of the method, and facilitates sample preparation and clean up. Often, stable derivatives can be formed from unstable metabolites. During derivatization, the samples are often dissolved in a buffer at pH as high as 9 and the buffer may also contain EDTA or similar compounds that chelate metal ions [49]. The fluorenylmethyloxycarbonyl chloride (FMOC) derivatization procedure effectively detected

BMAA in trial animals, cycads, and cycad flour [39] (Fig. 8.4a). It is possible to have two potential products of the derivatization reaction, either a single or double derivative (Fig. 8.4a). In two recent studies, BMAA was not detected in FMOC-derivatized preparations of human brain tissue, cyanobacteria, water samples, or supplements [35, 50].

A second derivatization technique uses the ethyl or propyl chloroformate derivative EZ:Faast [41, 42, 48] (Fig. 8.4b). In this method, propyl chloroformate is reacted with not only primary and secondary amines, but also with carboxyl groups in amino acids, with the derivative detectable by MS. When reacted with BMAA, this reagent generates a triply derivatized product with very different selectivity on C18 columns than other methods, making it an ideal system for orthogonal method comparisons [48].

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization as a method for quantification of BMAA has been verified by several orthogonal techniques [23, 51] including MS detection of the double derivative and monitoring for the single derivative peak [51, 52] (Fig. 8.4c). Successful AQC derivatization requires a pH of approximately 9, and acidic samples can fail to complete the reaction, leading to false negative results. Additionally, the borate buffer (Waters AccQ-Fluor Reagent) provided as part of the derivatization procedure contains a chelating agent that removes metal ions from the solution which may aid in derivatization and sample detection.

One of the biggest challenges with derivatization methods is ensuring that the reaction goes to completion. Partial derivatization or incomplete derivatization can fail to identify BMAA in samples and other amino acids such as norleucine or lysine can be useful for quantification of the efficiency of the derivatization reaction. This is particularly important when more than one reaction product is possible or when quantification of a minor metabolite in the presence of large excess of common reactants is required (Fig. 8.4a, c). BMAA is present in relatively small quantities in samples containing much larger amounts of the 20 protein amino acids, and the AQC derivatization reaction can result in two products with insufficient amounts of the reagent (Fig. 8.4c). Lysine also forms both single and double derivatized forms in the same reaction and can be used as a secondary internal standard to determine the completeness of the derivatization for each individual sample. The single derivatized form of lysine can be monitored by selected ion recording (SIR) of m/z 317.3 in MS systems or by the multiple-reaction-monitoring (MRM) transition m/z 317.3 → 171.0 in MS/MS instruments. The fragment m/z 171.0 results from loss of the AQC fluorophore tag from the parent ion and is a universal daughter ion in this method. When combined with its high ionization efficiency, this ubiquity makes it a good choice for MRM measurements for amino acids using this method. These data allow for monitoring and balancing of the derivatization reaction that is crucial to accuracy.

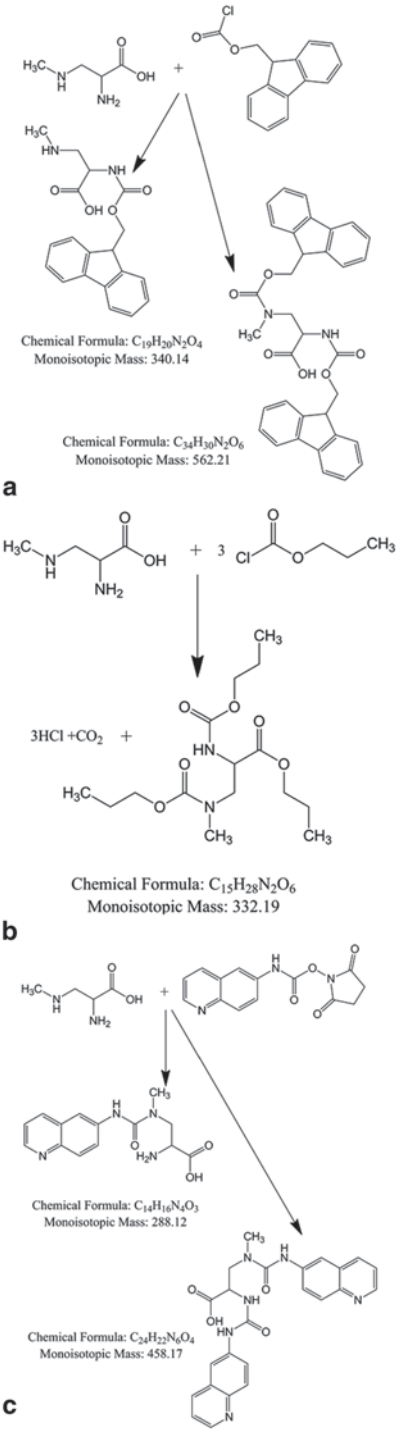


Fig. 8.4 Methods of derivatization of *β*–methyl-amino-alanine. **a** Fluorenyl-methyloxycarbonyl (FMOC) derivatives. **b** Propyl-chloroformate (EZ:faast) derivatives. **c** 6-Amino-quinolyl-N-hydroxysuccin-imidyl carbamate (AQC) derivatives

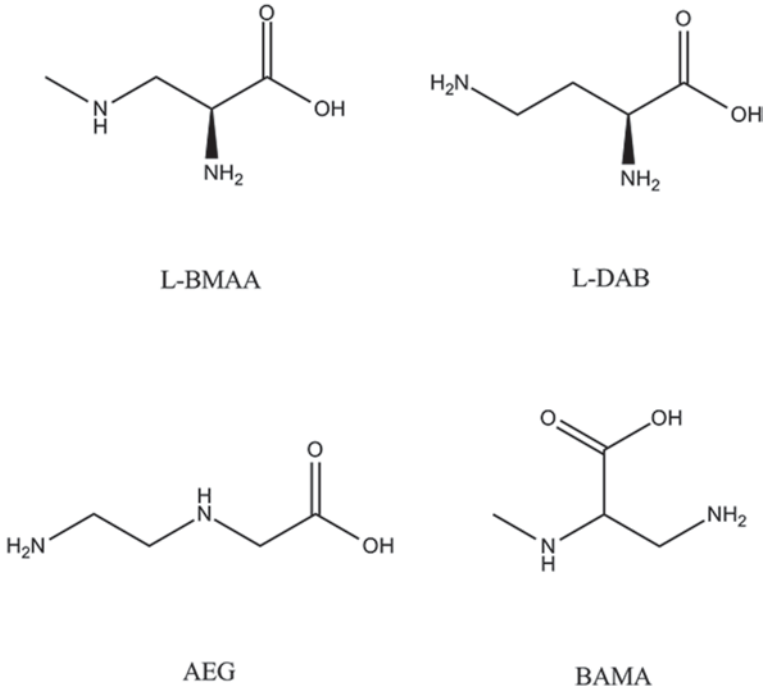


Fig. 8.5 Isomers of BMAA. *β*-Methylamino-L-alanine (BMAA), 2,4-diaminobutanoic acid (DAB), N-(2-aminoethy)glycine (AEG), *β*-amino-N-methylalanine (BAMA)

8.2.4 Chromatography

One of the most difficult problems of BMAA analysis in complex samples is the presence of isomers with identical molecular mass that need to be separated by chromatography (Fig. 8.5; [21, 52, 53]). From a database search of all the theoretical isomers of BMAA, a subset of seven possible interfering compounds were identified [53]. Two of these isomers, 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl) glycine (AEG), have been studied in detail since 2003 [21, 36, 37, 51–54]. Separation and identification of BMAA from DAB and AEG is achieved with derivatized samples through chromatographic separation (Fig. 8.6) [53–55].

The chemistry of the column and eluents are especially important for accurate BMAA analysis. Most of the reported separation systems used C_{18} columns but eight out of the nine reports that failed to detect BMAA in cyanobacteria used an HILIC column for separation and elution with formate/acetonitrile either isocratically or with a gradient (Table 8.1; reviewed in [33]).

A second approach uses hydrophilic interaction liquid chromatography (HILIC) to separate underivatized BMAA from the sample matrix [36–38, 46]. HILIC separations rely on the interactions between the polar stationary phase, liquid–liquid partitioning, ionic, and hydrophobic interactions with an initial elution in an organic

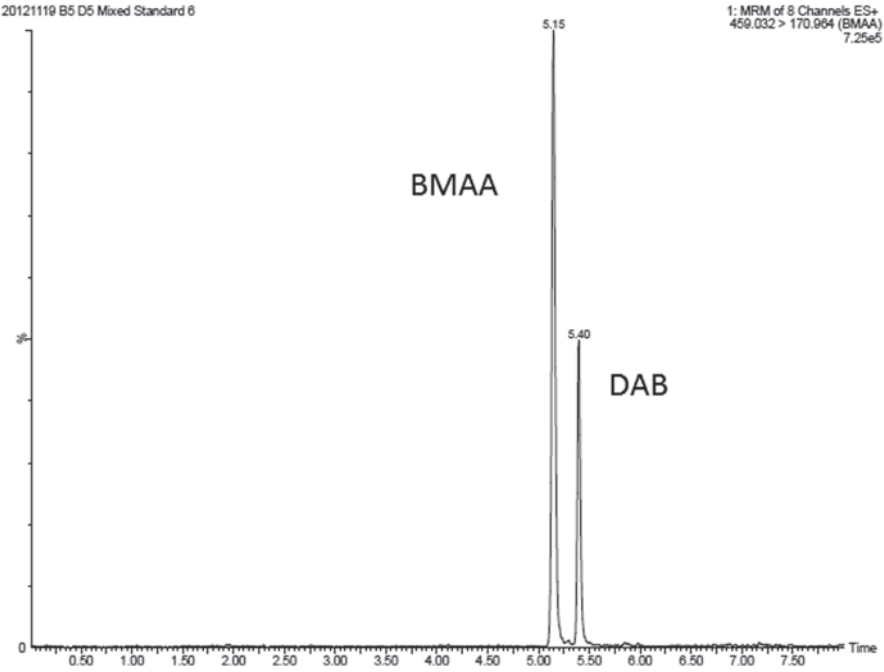


Fig. 8.6 Chromatographic separation of AQC-derivatized isomers BMAA and DAB on a Waters 100 × 2.1 mm, 1.7 μm C18 BEH column using a Waters Acquity I-Class UPLC. The flow rate was 0.65 mL/min with a column temperature of 30 °C. Compounds were eluted with 0.1 % formic acid (eluent A) and 0.1 % formic acid in acetonitrile (eluent B) and separated according to the following gradient: 0–0.5 min, 99.1 % A; 1.0–3.0 min, 95 % A; 5.5 min, 90 % A, curve 7; 8.0 min, 80 % A; 8.10–8.60 min, 15 % A; 9.0–10.0 min, 99.1 % A. A linear curve for each change in the gradient is assumed unless stated otherwise. BMAA and DAB were detected with a Waters Xevo TQ-S mass spectrometer in ESI + mode

solvent such as acetonitrile. One of the leading causes of peak distortion and signal loss in HILIC separations is the difference in viscosity and polarity between the sample and the organic elution buffer [56]. Samples for BMAA analysis were dissolved in acid [38, 56], water [36, 37], or a solution of acetonitrile and water [46]. The interaction of the polar sample diluents with the organic elution buffer may have reduced the solubility and separation of BMAA from the sample matrix [56].

8.2.5 Mass Spectrometry

Almost 70 % of the published literature relied on detection by MS (29 %) or MS/MS (40 %), while fluorescence detection (27 %) and diode array detection (4 %) proved less popular. Most LC-MS/MS methods have identified BMAA as a single underivatized parent ion, with the transition m/z 119 → 102 (loss of OH^-) [37, 43, 55] or a fragmentation pattern m/z 119 → 102, 88, 76, 73, and 44 [36] used for

quantification. Using a GC/GC-time of flight MS, Snyder et al. (2010) created a proprietary algorithm to combine signals of m/z 100, 101, 102, 103, 114, 115, 116, 117, 118, 129, 131, 132, 133, 291, and 292 to identify and quantify BMAA [42]. These approaches may have reduced quantitative accuracy in a complex matrix due to ion suppression arising from competition for available charge, surface saturation of ESI droplets with analytes, increase in droplet viscosity, and analyte hydrophobicity [33]. Recently, we demonstrated that overreliance on the underivatized BMAA precursor ion can lead to false negative results and that at least 30 BMAA adducts may be common in MS analysis (Table 8.2) [44]. The composition of the eluents and sample matrix contribute to the complexity of the adduct profile, and only 0–10% of the BMAA was represented by the [M+H]+peak. Other authors have argued that a D_3-BMAA internal standard "should be chelated with the metal to the same degree as BMAA, thus accounting efficiently for this effect" but this argument assumes that all reactions occur within the timeframe of the sample preparation from the addition of the D_3-BMAA through the extraction and injection [47]. Interestingly, a recent publication compared the concentrations obtained with AQC derivatization and fluorescence detection to those obtained by MS/MS analysis of the underivatized parent concluded "HPLC-FLD overestimated BMAA concentrations in some cyanobacterial samples" [55]. It is much more likely that the MS/MS analysis of the precursor and products ions underestimated BMAA as a result of adduct formation and ion suppression [33, 44]. Mass spectrometry can also be used to partially solve the problems of confounding results created by isomers of BMAA. Jiang et al. (2012) reported that only one of the possible BMAA isomers, *β*-amino-N-methylalanine (BAMA), produced the identical product ions as BMAA in tandem MS analysis of the AQC derivative but that the relative ratio of the daughters varied significantly [53] (Figs. 8.5 and 8.7).

8.3 Conclusions

Analysis of BMAA in natural samples is complicated by the polarity and reactivity of the molecule, the presence of BMAA at relatively low concentrations in complex sample matrices and the limitations of analytical instrumentation. Solid phase extraction media, liquid–liquid partitioning, and acid hydrolysis can be used to reduce the complexity of the sample matrix but the recovery of BMAA through each of these methods may be poor. Methods that rely on separation of BMAA from the sample matrix by HILIC are subject to interferences between the sample matrix, the sample diluent and the organic elution buffer that can mask the signal or obscure the chromatographic peak. Derivatization increases the size and reduces the reactivity of BMAA, thereby improving the separation chemistry and detection efficiency. Solvent interactions, ion suppression, and the formation of metal adducts and complexes can interfere with mass spectrometry identification and quantification. Advancements in our understandings of these areas will lead to better methods for identification and quantification of BMAA in biological and environmental samples and will help to resolve the ongoing controversy about the potential role(s) of BMAA in human health.

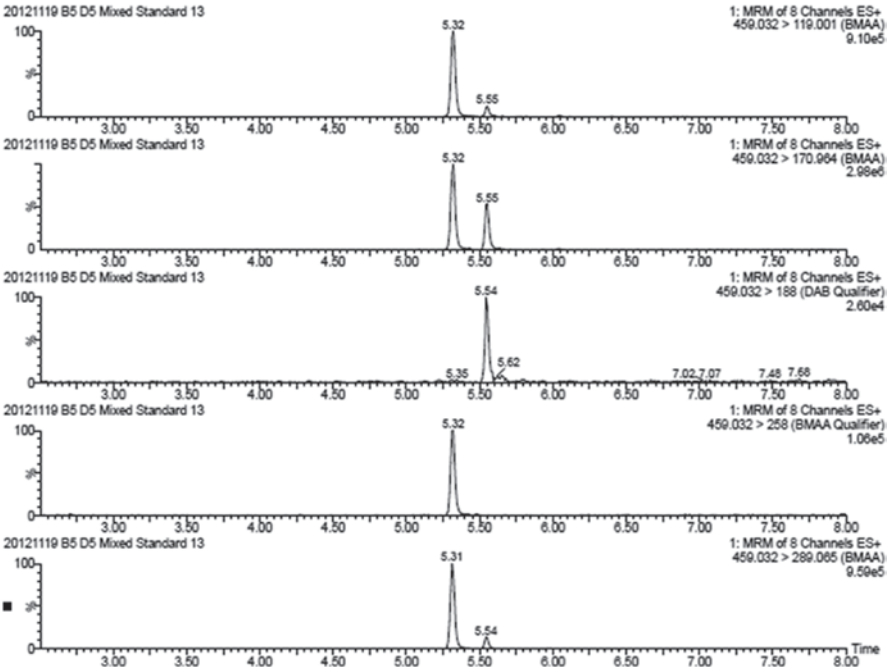


Fig. 8.7 Fragmentation of AQC derivatives of BMAA and DAB give characteristic daughter ions as qualifiers. **a** 459.0 → 119.0 transition resulting from loss of the fluorophore tags from both BMAA and DAB. **b** 459.0 → 171.0 fragmentation is seen in both BMAA and DAB. **c** 459.0 → 188.0 fragmentation is characteristic of DAB. **d** 459.0 → 258.0 fragmentation is characteristic of BMAA. **e** 459.0 → 289.1 fragmentation is seen in both BMAA and DAB. MRM transitions were scheduled to maximize the sensitivity of the mass spectrometer throughout the analysis. Two channels were monitored simultaneously from 0.0–3.0 min, both to ensure no single derivatized BMAA (189.0 → 171.0, CV 18), DAB (189.0 → 171.0, CV 18) or lysine (317.3 → 171.0, CV 20) were present in the sample. From 2.50 to 8.0 min, five MRM channels were used. Three were used for both BMAA and DAB: 459.0 → 119.0, CV 20; 459.0 → 171.0, CV 30; and 459.0 → 289.1, CV 14. Two additional channels were used as qualifiers for BMAA (459.0 → 258.0, CV 20) and DAB (459.0 → 188.0, CV 20) as these transitions are specific to each AQC-derivatized molecule. Double-derivatized lysine (487.2 → 171.0, CV 18) was monitored from 6.0–8.0 min to ensure derivatization completeness and prevent the occurrence of false negatives in the quantification process. A cone voltage of 16 V and a dwell time of 60 ms were used for all transitions monitored

References

1. Arnold A, Edgren DC, Paladino VS (1953) Amyotrophic lateral sclerosis: fifty cases observed on Guam. J Nerv Ment Dis 117:135–139
2. Koerner DR (1952) Amyotrophic lateral sclerosis on Guam: a clinical study and review of the literature. Ann Int Med 37:1204–1220
3. Tillema S, Wijnberg CJ (1953) 'Endemic' amyotrophic lateral sclerosis on Guam: epidemiological data; a preliminary report. Docum Med Geog Et Trop 5:366–370
4. Kurland LT, Mulder DW (1954) Epidemiologic investigations of amyotrophic lateral sclerosis. 1. Preliminary report on geographic distribution with special reference to the Mariana Islands, including clinical and pathologic observations. Neurology 4:355–378

5. Kurland LT, Mulder DW (1954) Epidemiologic investigations of amyotrophic lateral sclerosis. 1. Preliminary report on geographic distribution with special reference to the Mariana Islands, including clinical and pathologic observations. Neurology 4:438–448
6. Reed W, Plato C, Elizan T, Kurland LT (1966) The amyotrophic lateral sclerosis/parkinsonism-dementia complex: a ten year follow up on Guam. Am J Epidem 83:54–73
7. Reed D, Torres JM, Brody JA (1975) Amyotrophic lateral sclerosis and parkinsonism dementia on Guam 1945–1972. Am J Epidem 101:302–310
8. Plato CC, Reed DM, Elizan TS, Kurland LT (1967) Amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam. Am J Hum Gen 19:617–632
9. Kurland LT, Mulder DW (1955) Epidemiologic Investigations of amyotrophic lateral sclerosis. 2. Familial aggregations indicative of dominant inheritance. Neurology 5:182–196
10. Kurland LT, Mulder DW (1955) Epidemiologic Investigations of amyotrophic lateral sclerosis. 2. Familial aggregations indicative of dominant inheritance. Neurology 5:249–268
11. Reed D, Labarthe D, Chen KM, Stallones R (1987) A cohort study of amyotrophic lateral sclerosis and parkinsonism dementia on Guam and Rota. Am J Epidem 125:92–100
12. Whiting MJ (1963) Toxicity of cycads. Econ Bot 17:270–302
13. Laqueur GL, Mickelsen O, Whiting MG, Kurland LT (1963) Carcinogenic properties of nuts from *Cycas circinalis* L. indigenous to Guam. J Nat Cancer Inst 31:919–951
14. Vega A, Bell EA (1967) α -Amino-methylamiopropionic acid, a new amino acid from seeds of *Cycas circinalis*. Phytochem 6:759–762
15. Vega A, Bell EA, Nunn PB (1968) The preparation of L- and D-α - amino-β -methylaminopropionic acids and the identification of the compound isolated from *Cycas circinalis* as the L-isomer. Phytochem 7:1885–1887
16. Spencer PS, Nunn PB, Hugon J, Ludolph AC, Ross SM, Roy DN, Robertson RC (1987) Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin. Science 237:517–522
17. Duncan MW, Kopin IJ, Garruto RM, Lavine L, Markey SP (1988) 2-Amino-3(methylamino)-propionic acid in cycad-derived foods is an unlikely cause of amyotrophic lateral sclerosis/parkinsonism. The Lancet 2:631–632
18. Duncan MW, Steele JC, Kopin IJ, Markey SP (1990) 2-Amino-3-(methylamino)-propionic acid (BMAA) in cycad flour: an unlikely cause of amyotrophic lateral sclerosis and parkinsonism-dementia of Guam. Neurology 40:767–772
19. Duncan MW, Marini AM, Wattem R, Kopin IJ, Markey SP (1992) Zinc, a neurotoxin to cultured neurons, contaminates cycad flour prepared by traditional Guamanian methods. J Neurosci 12:1523–1537
20. Cox PA, Sacks OW (2002) Cycad neurotoxins, consumption of flying foxes, and ALS-PDC disease in Guam. Neurology 58:956–959
21. Banack SA, Cox PA (2003) Distribution of the neurotoxic nonprotein amino acid BMAA in *Cycas micronesica*. Bot J Linnean Soc 143:165–168
22. Cox PA, Banack SA, Murch SJ (2003) Biomagnification of cyanobacterial neurotoxins through the ecosystem: implications for ALS-PDC in Guam. Proc Natl Acad Sci U S A 100:13380–13383
23. Murch SJ, Banack SB, Cox PA (2004) A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. Proc Natl Acad Sci U S A 101:12228–12231
24. Banack SA, Murch SJ, Cox PA (2006) Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Islands. J Ethnopharm 106:97–104
25. Banack SA, Murch SJ (2009) Multiple neurotoxic items in the Chamorro diet link BMAA with ALS/PDC. Amyotroph Lateral Scler 10:34–40
26. Murch SJ, Cox PA, Banack SA, Steele JC, Sacks OW (2004) Occurrence of β-methylamino-l-alanine (BMAA) in ALS/PDC patients from Guam. Acta Neurologica Scand 110:267–269
27. Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS, Morrison LF, Codd GA, Bergman B (2005) Diverse taxa of cyanobacteria produce beta-N-methylamino-L-alanine, a neurotoxic amino acid. Proc Natl Acad Sci U S A 102:5074–5078

28. Brandt LE, Pablo J, Compton A, Hammerschlag N, Mash DC (2010) Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-L-alanine (BMAA), in South Florida aquatic food webs. Harmful Algae 9:620–635
29. Jonasson S, Eriksson J, Berntzon L, Spácil Z, Ilag LL, Ronnevi L-O, Rasmussen U, Bergman B (2010) Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc Natl Acad Sci U S A 107:9252–9257
30. Roney BR, Renhui L, Banack SA, Murch SJ, Honegger R, Cox PA (2009) Consumption of fa cai Nostoc soup and potential BMAA exposures in China. Amyotroph Lateral Scler 10:44–49
31. Mondo K, Hammerschlag N, Basile M, Pablo J, Banack SA, Mash DC (2012) Cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) in shark fins. Marine Drugs 10:509–520
32. Pablo J, Banack SA, Cox PA, Johnson TE, Papapetropoulos S, Bradley WG, Buck A, Mash DC (2009) Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. Acta Neuro Scand 120:216–225
33. Cohen SA (2012) Analytical techniques for the detection of a-amino-b-methylamino-propionic acid. Analyst 137:1991–2005
34. Duncan MW (2012) Good mass spectrometry and its place in good science. J Mass Spec 47:795–809
35. Montine TJ, Li K, Perl DP, Galasko D (2005) Lack of beta-methylamino-L-alanine in brain from controls, AD, or Chamorros with PDC. Neurology 65:768–769
36. Rosen J, Hellenas KE (2008) Determination of the neurotoxin BMAA (β-N-methylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry). Analyst 133:1785–1789
37. Kruger T, Monch B, Oppenhauser S, Luckas B (2010) LC-MS/MS determination of the isomeric neurotoxins BMAA (beta-N-methylamino-L-alanine) and DAB (2,4-diaminobutyric acid) in cyanobacteria and seeds of *Cycas revoluta* and *Lathyrus latifolius*. Toxicon 55:547–557
38. Li A, Fan H, Ma F, McCarron P, Thomas K, Tanga X, Quilliam MA (2012) Elucidation of matrix effects and performance of solid-phase extraction for LC-MS/MS analysis of b-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB) neurotoxins in cyanobacteria. Analyst 137:1210–1219
39. Kisby GE, Roy DN, Spencer PS (1988) Determination of β-N-methylamino-L-alanine (BMAA) in plant (*Cycas circinalis* L.) and animal tissue by precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC) and reversed-phase high-performance liquid chromatography. J Neurosci Meth 26:45–54
40. Esterhuizen M, Downing TG (2008) β-N-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicol Environ Saf 71:309–313
41. Esterhuizen-Londt M, Downing S, Downing TG (2011) Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-N-methylamino-L-alanine (BMAA) in cyanobacteria. Water SA 37:133–138
42. Snyder LR, Hoggard JC, Montine TJ, Synovec RE (2010) Development and application of a comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry method for the analysis of L-beta-methylamino-alanine in human tissue. J Chromatogr A 27:4639–4647
43. Faassen EJ, Grillissen F, Zweers HAJ, Lurling M (2009) Determination of the neurotoxins BMAA (β-N-methylamino-L-alanine) and DAB (α-,γ-diaminobutyric acid) by LC-MSMS in Dutch urban waters with cyanobacterial blooms. Amyotroph Lateral Scler 10:79–84
44. Glover WB, Liberto CM, McNeil WS, Banack SA, Shipley PR, Murch SJ (2012) Reactivity of β-methylamino-L-alanine in complex sample matrices complicates detection and quantification by mass spectrometry. Anal Chem 84:7946–7953
45. Khabazian I, Bains JS, Williams DE, Cheung J, Wilson JM, Pasqualotto BA, Pelech SL, Andersen R. J, Wang YT, Liu L, Nagai A, Kim SU, Craig UK, Shaw CA (2002). J Neurochem 82:516–528
46. Kubo T, Kato N, Hosoya K, Kaya K (2008) Effective determination method for a cyanobacterial neurotoxin, beta-N-methylamino-L-alanine. Toxicon 51:1264–1268

47. Jiang L, Johnston E, Åberg KM, Nilsson U, Ilag LL (2013) Strategy for quantifying trace levels of BMAA in cyanobacteria by LC/MS/MS. Anal Bioanal Chem 405:1283–1292
48. Downing S, Banack SA, Metcalf JS, Cox PA, Downing TG (2011) Nitrogen starvation of cyanobacteria results in the production of b-N-methylamino-L-alanine. Toxicon 58:187–196
49. Rebane R, Herodes K (2012) Comparison of three buffer solutions for amino acid derivatization and following analysis by liquid chromatography electrospray mass spectrometry. J Chromatogr A 1245:134–142
50. Scott PM, Niedzwiadek B, Rawn DEK, Lau BPY (2009) Liquid chromatographic determination of the cyanobacterial toxin β-N-methylamino-L-alanine in algae food supplements, freshwater fish, and bottled water. J Food Prot 72:1769–1773
51. Banack SA, Metcalf JS, Spácil Z, Downing TG, Downing S, Long S, Nunn PB, Cox PA (2011) Distinguishing the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) from other diamino acids. Toxicon 57:730–738
52. Banack SA, Downing TG, Spácil Z, Purdie EL, Metcalf JS, Downing S, Esterhuizen M, Codd GA, Cox PA (2010) Distinguishing the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). Toxicon 56:868–879
53. Jiang L, Aigret B, De Borggraeve WM, Spacil Z, Ilag LL (2012) Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples. Anal Bioanal Chem 403:1719–1730
54. Banack SA, Metcalf JS, Jiang L, Craighead D, Ilag LL, Cox PA (2012) Cyanobacteria produce N-(2-aminoethyl)glycine, a backbone for peptide nucleic acids which may have been the first genetic molecules for life on earth. PLoSOne 7:e49043
55. Faassen EJ, Gillissen F, Lurling M (2012) A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria. PLoSOne 7:e36667
56. Grumbach ES, Fountain KJ (2010) Comprehensive guide to HILIC: hydrophilic Interaction Chromatogrphy. Waters, Milford Massachusetts. pp 41–44
57. Banack SA, Johnson HE, Cheng R, Cox PA (2007) Production of the neurotoxin BMAA by a marine cyanobacterium. Mar Drugs 5:180–196
58. Johnson HE, King SR, Banack SA, Webster C, Callanaupa WJ, Cox PA (2008) Cyanobacteria (Nostoc commune) used as a dietary item in the Peruvian highlands produce the neurotoxic amino acid BMAA. J Ethnopharm 118:159–165
59. Metcalf JS, Banack SA, Lindsay J, Morrison LF, Cox PA, Codd GA (2008) Co-occurrence of beta-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990–2004. Environ Microbiol 10:702–708
60. Bidigare RR, Christensen SJ, Wilde SB, Banack SA (2009) Cyanobacteria and BMAA: possible linkage with avian vacuolar myelinopathy (AVM) in the south-eastern United States. Amyotroph Lateral Scler 10:71–73
61. Eriksson J, Jonasson S, Papaefthimiou D, Rasmussen U, Bergman B (2009) Improving derivatization efficiency of BMAA utilizing AccQ-Tag in a complex cyanobacterial matrix. Amino Acids 36:43–48
62. Cox PA, Richer R, Metcalf JS, Banack SA, Codd GA, Bradley WG (2009) Cyanobacteria and BMAA exposure from desert dust: a possible link to sporadic ALS among Gulf War veterans. Amyotroph Lateral Scler 10:109–117
63. Craighead D, Metcalf JS, Banack SA, Amgalan L, Reynolds HV, Batmunkh M (2009) Presence of the neurotoxic amino acids beta-N-methylamino-L-alanine (BMAA) and 2,4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert. Amyotroph Lateral Scler 10:96–100
64. Li A, Tian Z, Li J, Yu RC, Banack SA, Wang ZY (2010) Detection of the neurotoxin BMAA within cyanobacteria isolated from freshwater in China. Toxicon 55:947–953
65. Sp!acil Z, Eriksson J, Jonasson S, Rasmussen U, Ilag LL, Bergman B (2010) Analytical protocol for identification of BMAA and DAB in biological samples. Analyst 135:127–132
66. Cianca RCC, Baptista MS, Lopes VR, Vasconcelos VM (2012) The non-protein amino acid b-N-methylamino-L-alanine in Portuguese cyanobacterial isolates. Amino Acids 42:2473–2479

67. Cianca RCC, Baptista MS, Pinto da Silva L, Lopes VR, Vasconcelos VM (2012) Reversed-phase HPLC/FD method for the quantitative analysis of the neurotoxin BMAA (b-N-methyl-amino-L-alanine) in cyanobacteria. Toxicon 59:379–384
68. Metcalf JS, Banack SA, Kotut K, Krientiz L, Codd GA (2012) Amino acid neurotoxins of the lesser flamingo, Phoeniconaias minor. Chemosphere 90:836–839
69. Huang N, Siegel MM, Kruppa GH, Laukien FH (1999) Automation of a Fourier transform ion cyclotron resonance mass spectrometer for acquisition, analysis, and e-mailing of high-resolution exact-mass electrospray ionization mass spectral data. J Am Soc Mass Spectrom 10:1166–1173

Index

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application*,
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5